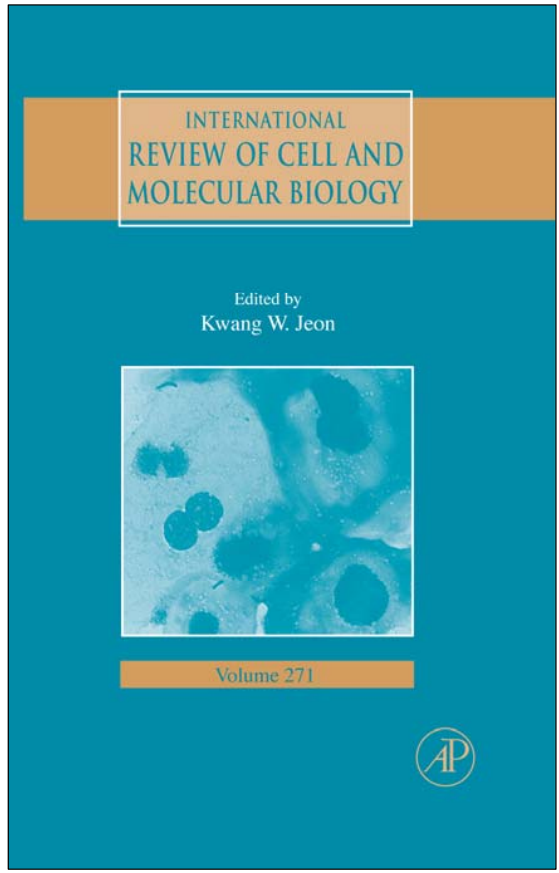
Phagocytosis and host pathogen interactions in Dictyostelium with a look at macrophages



From: Salvatore Bozzaro, Cecilia Bucci, and Michael Steinert, Phagocytosis and Host–Pathogen Interactions in *Dictyostelium* with a Look at Macrophages. In Kwang W. Jeon, editor: International Review of Cell and Molecular Biology, Vol. 271, Burlington: Academic Press, 2008, pp. 253-300.
ISBN: 978-0-12-374728-0

CHAPTER SIX

Phagocytosis and Host–Pathogen Interactions in *Dictyostelium* with a Look at Macrophages

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International Review of Cell and Molecular Biology, Volume 271
ISSN 1937-6448, DOI: 10.1016/S1937-6448(08)01206-9

Abstract

Research into phagocytosis and host–pathogen interactions in the lower eukaryote *Dictyostelium discoideum* has flourished in recent years. This chapter presents a glimpse of where this research stands, with emphasis on the cell biology of the phagocytic process and on the wealth of molecular genetic data that have been gathered. The basic mechanistic machinery and most of the underlying genes appear to be evolutionarily conserved, reflecting the fact that phagocytosis arose as an efficient way to ingest food in single protozoan cells devoid of a rigid cell wall. In spite of some differences, the signal transduction pathways regulating phagosome biogenesis are also emerging as ultimately similar between *Dictyostelium* and macrophages. Both cell types are hosts for many pathogenic invasive bacteria, which exploit phagocytosis to grow intracellularly. We present an overwiew, based on the analysis of mutants, on how *Dictyostelium* contributes as a genetic model system to decipher the complexity of host–pathogen interactions.

Key Words: *Dictyostelium*, *Legionella*, *Mycobacteria*, *Klebsiella*, macrophages, phagocytosis, host–pathogen interactions, cytoskeleton, signal transduction, phosphoinositides, G proteins, Nramp.

1. Introduction

Phagocytosis is a process initiated by binding of the particle to the cell surface, its progressive surrounding by the plasma membrane and ingestion of the newly produced vesicle, called the phagosome. The phagosome undergoes maturation by fusing with vesicles of the endocytic pathway and by gradually acquiring properties typical of the lysosome. In metazoa, with a developed immune system, phagocytosis is a feature of specialized, ''professional phagocytes'' (macrophages, neutrophils, and dendritic cells), which are capable of ingesting and killing a large variety of microorganisms (Haas, 2007; Stuart and Ezekowitz, 2005). In addition to this protective function against microbes, phagocytosis plays a crucial role in noninflammatory depletion of apoptotic cells, thus in tissue remodeling and development.

A few pathogenic microbes exploit the phagocytic pathway to invade the cells and, by escaping digestion, cause different sorts of infections (Celli and Finlay, 2002; Gruenberg and van der Goot, 2006; Mueller and Pieters, 2006). Infectious diseases represent a serious health threat, being the first cause of mortality in the world, further complicated by the emergence of various resistant microbe strains associated to multidrug resistance. A thorough knowledge of the subcellular mechanisms underlying phagocytosis, and their subversion by invasive microbes, may help in designing more effective thrapeutic approaches to fight intracellular pathogens. Of great

help for such understanding is the development of model organisms amenable to genetic manipulation that facilitate identification and analysis of genes regulating phagocytosis and modulating host resistance to pathogens. The awareness that phagocytosis is evolutionary very ancient, being already present in unicellular amoebae, has favored in recent years the establishment of a few such models, like *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster* (Pradel and Ewbank, 2004; Stuart and Ezekowitz, 2008).

Dictyostelium cells, like all ''social amoebae'' or ''cellular slime molds,'' live in the forest soil and feed on bacteria. As long as bacteria are available, the solitary amoebae ingest them, grow and proliferate by binary fission. When the bacteria are depleted, the cells stop growing and enter the social phase of their life cycle, by collecting into multicellular aggregates, which like all multicellular organisms undergo cell differentiation and morphogenesis. The outcome is a fruiting body, consisting of a slender stalk bearing on top a ball of resistant spores (Kessin, 2001).

The phagocytic totipotentiality of growth phase cells gradually declines when cells enter the multicellular stage and form tight aggregates. When exposed to bacteria, starving preaggregating or aggregating cells fully revert to the phagocytic stage, but after aggregate compaction less than 20% of the cells in the aggregate display the ability to phagocytose and grow on bacteria (Gambino *et al.*, 1992). At the migrating slug stage, this number is further reduced to about 1%, and evidence has been recently provided that these cells, named ''sentinel cells,'' may represent a reservoir of immune-like cells that circulate within the slug, swallow invading bacteria and toxic substances, and are left behind the slug during its migration (Chen *et al.*, 2007). The idea of a primitive innate immunity system active during multicellular development is further supported by the occurrence in the *Dictyostelium* genome of potential homologues to innate immunity signaling proteins found in animals or plants, including Toll-like Interleukin Receptor (TIR)-domain containing proteins, WRKY transcription factors, potential LRR-domain receptors (Chen *et al.*, 2007).

Vegetative *Dictyostelium* cells are highly efficient phagocytes, and their capacity exceeds that of neutrophils severalfold, each cell being able to digest about 300 bacteria per hour. The cells are considered ''professional phagocytes,'' as they ingest a large variety of bacteria, yeast, apoptotic cells, as well as inert particles. In contrast, the various species of social amoebae usually do not swallow one another, thus displaying a high degree of self-non-self recognition. A cannibalistic species was described, *Dictyostelium caveatum*, which is able to eat its neighbors, when cocultured with other species. Interestingly, *D. caveatum* cells do not devour each other, except for a mutant that has completely lost self-non-self recognition (Waddell and Duffy, 1986). Unfortunately, very little is known on the genetic background of *D. caveatum*.

Phagocytosis has been mostly studied in the species *D. discoideum*, which is used here synonymously as *Dictyostelium*, except when otherwise indicated. For the molecular analysis of phagocytosis, a breakthrough was the isolation of laboratory axenic strains that grow in minimal or rich axenic media (see Kessin, 2001). In contrast to the wild-type strains, which are strictly dependent on bacterial phagocytosis for growth, the axenic strains have gained the function of swallowing nutrients by fluid-phase endocytosis, without loosing the ability to phagocytose. These strains are thus ideal for a genetic approach to phagocytosis, as mutants defective in phagocytosis, but able to grow by fluid-phase endocytosis, can be selected.

With the establishment in the course of the last 25 years of molecular genetic techniques for transforming cells, disrupting single genes and randomly generating mutants by restriction enzyme-mediated insertion, the shortcomings of asexual *Dictyostelium* genetics could be overcome, making the system amenable to molecular genetic manipulation. The recent completion of genome sequencing has made available new tools for postgenomic analysis of phagocytosis (Eichinger *et al.*, 2005; Eichinger and Rivero, 2006).

The relevance of *Dictyostelium* as model system for phagocytosis and for immunity is highlighted by the broad spectrum of bacteria that are taken up by the cells, and by its susceptibility to infection by microbes that are pathogenic also for animals and humans. A first systematic attempt to identify pathogenic bacteria for *Dictyostelium* can be traced back to a paper by Depraitère and Darmon (1978). These authors tested 45 Gram-negative and 23 Gram-positive bacterial species for their capacity to support *Dictyostelium* cell growth, and found that the cells were able to ingest and degrade most of them, including several species of *Enterobacter, Serratia, Salmonella, Klebsiella, Yersinia, Pseudomonas, Staphylococcus, Listeria*, and several *Bacilli*. A few species or strains, such as highly pigmented *Serratia marcescens* strains, *Pseudomonas aeruginosa*, *Pseudomonas cepacia, Pseudomonas rubescens, Bacillus thuringiensis*, *Bacillus laterosporus, and Listeria monocytogenes*, were found to be pathogenic. Except for *P. aeruginosa* and *Bacillus thuringiensis*, which killed the cells by secreting toxic substances, the other bacteria were phagocytosed and were toxic at different degree for *Dictyostelium* cells (Depraitère and Darmon, 1978). These authors also reported that *B. anthracis* was not ingested by *Dictyostelium* cells. Recent studies have detected other pathogenic microbes and have shown that host–pathogen interactions can be studied at molecular level in *Dictyostelium*, by exploiting all the advantages of a lower eukaryote easily amenable to molecular genetic, biochemical, and cell biology investigations (see Section 5).

Both fluid-phase endocytosis and phagocytosis in *Dictyostelium* have been reviewed in recent years (Duhon and Cardelli, 2002; Maniak, 2002). Host–pathogen interactions have been the subject of sectorial reviews (Solomon and Isberg, 2000; Steinert and Heuner, 2005). In this chapter,

we will reexamine both phagocytosis and host–pathogen interactions in *Dictyostelium*, in the light of recent developments in both fields. When appropriate, comparisons with results on mammalian macrophages will be made. Fluid-phase endocytosis and macropinocytosis will not be discussed, and the readers are invited to look at the excellent reviews by Maniak (2002, 2001).

2. The Dynamics of Phagocytosis

Several studies have made use of cells expressing GFP-fused proteins and fluorescently-labeled bacteria, latex beads or yeast particles to dissect the dynamics of phagocytosis *in vivo*. In these studies, cells and particles are coincubated on glass, to follow both the amoeboid movements of the cell in its attempt to hold and encapsulate the prey and the intracellular fate of the ingested phagosome. Alternatively, coincubation in shaken culture has been used in pulse-chase experiments for quantitative measurements of uptake and intracellular phagosome maturation or for isolating phagosomal populations for proteomic or transcriptomic studies.

In a highly simplified scheme, it is assumed that particle binding leads to progressive actin recruitment at the site of attachment and along the rim of the membrane enveloping the particle, leading eventually to closure of the phagocytic cup around the particle. Formation of the phagocytic cup resembles, thus, pseudopod formation during chemotaxis, as in both cases the actin cytoskeleton drives a local membrane extension (Fig. 6.1). Occasionally, "sinking" of bacteria into the cell has been observed, a process that possibly requires a different rearrangement of the underlying cytoskeleton (Fig. 6.1).

A basic difference between a chemotactic pseudopod and a phagocytic cup is that the first is better explained with a trigger model and the second with a zipper model. In the trigger model, a chemotactic signal initiates an all-or-none response, leading to pseudopod formation. In the zipper model, phagocytosis is assumed to be locally controlled by progressive, sequential binding of cell surface receptors to the particle, a process that can be interrupted at any moment (Swanson and Baer, 1995). Evidence for the zipper model has been provided in *Dictyostelium*, using yeast particle as prey. It has been shown that particle binding does not necessarily lead to uptake, and that about half of the attempts are aborted, though actin is locally recruited. Even formation of a leading pseudopod in front of the particle or prolonged binding is no guarantee of success (Maniak *et al.*, 1995; Peracino *et al.*, 1998). These early studies were done with yeasts, which are 3–5 μm in diameter. Similar studies with bacteria are difficult to perform, due to flagellar-induced or brownian motion of the bacteria.

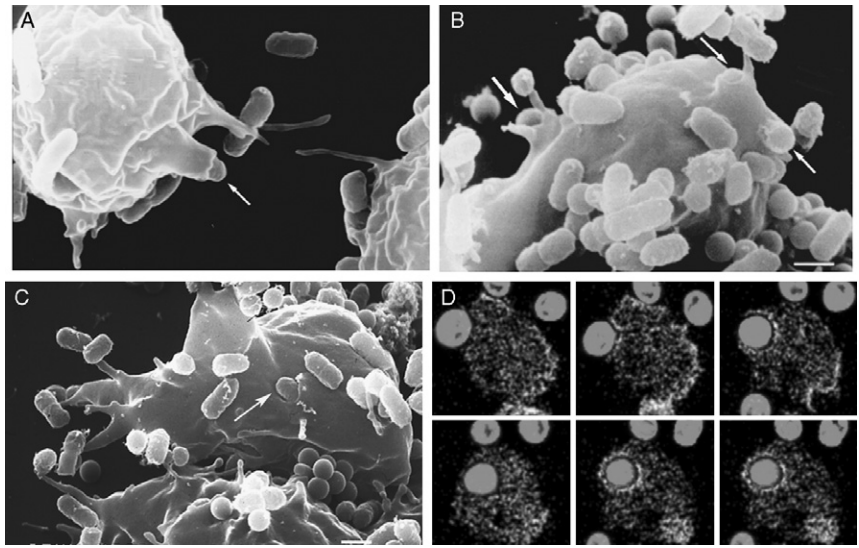

Figure 6.1 Phagocytosis of bacteria by *Dictyostelium* cells. (A–C) Scanning electron micrographs of *Dictyostelium* cells incubated with *E. coli* B/r. (A, B) Typical examples of pseudopod-like phagocytic cups with bacteria inside are indicated (arrows). (C) The arrow points to a bacterium "sinking" into the cell. The round particles are latex beads. (D, clockwise) Confocal image series of a living cell, expressing GFP-actin, ingesting a yeast particle. The time elapsing from actin recruitment to the phagocytic cup to its disappearance from the ingested particle takes about 1 min. (SEM in collaboration with the late M. Claviez, confocal fluorescence microscopy with B. Peracino.)

Alternative approaches with bacteria or latex beads have made use of the particle-tracking method, to reduce particle movement and favor adhesion (Ishikawa *et al.*, 2003), or the agar overlay technique, where a thin agar sheet gently pressing the cells creates a ''two-dimensional'' condition, in which the bacteria are mostly immobile and the cell ingests them by extending large pseudopodia around (Clarke and Maddera, 2006). Also under these conditions, it has been qualitatively or semiquantitatively shown that attached bacteria or beads are often not phagocytosed.

Successful phagocytosis events can be, however, very rapid. Both with yeasts and bacteria, the time elapsing from initial actin recruitment at the phagocytic cup to disassembly of the actin coat around the phagosome can take as less as 60 s (Blanc *et al.*, 2005; Clarke and Maddera, 2006; Dormann *et al.*, 2004; Insall *et al.*, 2001; Loovers *et al.*, 2007; Maniak *et al.*, 1995; Peracino *et al.*, 1998). The ingested phagosome undergoes successive fusion events with vesicles of the endolysosomal compartment, resulting in acidification, delivery of a large variety of hydrolases, postlysosomal maturation and eventually fusion of the ''postlysosomal'' vesicle with the plasma membrane for excreting undigested debris. From this point of view, phagocytosis in *Dictyostelium* is considered a linear process, starting with uptake and

ending with excretion of undigested material, though retrieval and recycling of several membrane components has been shown to occur from the very early steps upward (Gotthardt *et al.*, 2002; Ravanel *et al.*, 2001).

Fusion of the phagosome with acidic vesicles, visualized by using pH-sensitive probes, such as neutral red or Lysotracker red, or GFP-fused subunits of the vacuolar H^+ ATPase (the major agent responsible for acidification) is a rapid event, detectable within 1–2 min after phagosome formation (Clarke and Maddera, 2006; Maniak, 2001; Peracino *et al.*, 2006; Rupper *et al.*, 2001a). In contrast to macrophages, acidification is a transitory event: in pulse-chase experiments, it has been shown that phagosomes, as well as macropinosomes, become acidic (pH 5.0) within 5 min, then the pH gradually increases to about 6.0 between 10 and 60 min and above 6.0 thereafter (Aubry *et al.*, 1993; Padh *et al.*, 1993; Rupper and Cardelli, 2001). Neutralization of the pH is linked to retrieval of the vacuolar H^+ ATPase (Aubry *et al.*, 1993; Clarke *et al.*, 2002a).

Neutralization is also accompanied by shape changes of the phagosome. The nascent phagosome is characterized by tight apposition of the phagosomal membrane with the ingested particle. Between 15 and 60 min postuptake, there is an increase in the number of phagosomes containing multiple bacteria, suggesting homotypic fusion between phagosomes. Later on, the multiparticle phagosomes become swollen, giving rise to so-called ''spacious phagosomes,'' large vacuoles in which degraded bacteria are visible (Rauchenberger *et al.*, 1997; Rupper *et al.*, 2001b). At the level of single cell phagocytosing few bacteria, digestion has been calculated to be accomplished in 4 min from ingestion (Clarke and Maddera, 2006), though at population levels longer times, averaging 20 min, have been calculated (Aubry *et al.*, 1993; Clarke *et al.*, 2002a; Maselli *et al.*, 2002). Release of undigested debris is instead a slow process, going on over several hours postuptake.

Phagocytosis in shaken cocultures with bacteria is usually studied by using a 100- to 1000-fold excess of fresh bacteria in simple salt solution, in which the bacteria cannot grow. Under optimal conditions, cells ingest about 1000 bacteria per generation (3 h), with an uptake rate of 4–5 bacteria per minute (Bozzaro and Gerisch, 1978; Gerisch, 1959; Vogel *et al.*, 1980). Pulse-chase experiments with bacteria or latex beads under similar conditions have been designed for isolation and proteomic characterization of purified phagosomes during their maturation (Bogdanovic *et al.*, 2002; Gotthardt *et al.*, 2002, 2006; Rezabek *et al.*, 1997). These studies have confirmed very early recruitment of the V-H^+ATPase in the phagosome, concomitantly with shedding of the actin coat and delivery of proteins regulating vesicle fusion, such as the small G protein Rab7, the SNARE components Vti1, syntaxin 7, syntaxin 8, and the lysosomal marker LmpB. A second step in the maturation of phagosomes, starting between 3 and 15 min postuptake, is characterized by recruitment of lysosomal enzymes, such as cathepsin D and cysteine proteinases

CP-p34, together with lysosomal membrane markers, such as the CD36/LIMP family members lmpA and lmpC, and the SNARE component Vamp7. Slightly delayed is the acquisition of lysosomal glycosidases, such as α-mannosidase and β-glucosidase. Acquisition of postlysosomal markers, such as the vacuolin B protein, is a late event, occurring 60–90 min postinternalization (Rauchenberger *et al.*, 1997).

The maturation process is accompanied by extensive retrieval and recycling, which occur with different kinetics for different markers, confirming the occurrence of complex and successive fusion events (Gotthardt *et al.*, 2002). Discrepancies in the reported kinetics of delivery, but in particular recycling, of proteins depend on whether living bacteria or latex beads are used as particles, very likely because bacteria are degraded while latex beads are indigestible inert particles. The timing of uptake and phagosome formation is similar for both particles, but subsequent phagosome maturation is slowed down for the latex beads. Toxic effects of the engulfed beads, particularly at high concentrations, cannot be excluded, though cell lysis is rarely observed, and the cells fully recover after latex bead excretion.

In addition to the above-mentioned proteins, characterization of the phagosomal proteome has led to identification of about 180 proteins, most of which are involved in membrane traffic, metabolism, signal transduction, protein biosynthesis, and degradation (Gotthardt *et al.*, 2006). Proteomic fingerprinting of the phagosome has confirmed substantial remodeling during the different phases of phagocytosis, with major changes occurring shortly after uptake and at the postlysosomal exocytic stage. A preliminary lipidomic characterization of early phagosomes compared to the plasma membrane has revealed a significant shift from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species toward lyso-PC and -PE and toward phospholipids with shorter acyl chains and lower unsaturations (Gotthardt *et al.*, 2006). Enrichment of sterols in the phagosomal membrane, compared to the bulk of plasma membrane, was previously reported (Favard-Sereno *et al.*, 1981). Consistent with these results is the recent observation in microarray studies that phagocytosis induces down- and upregulation of genes involved in phospholipid and sterol biosynthesis, respectively (Sillo *et al.*, 2008).

3. Cellular Mechanisms of Phagocytosis

3.1. Bacterial adhesion to the cell surface: The search for phagocytosis receptors

As just mentioned, *Dictyostelium* cells ingest bacteria very efficiently, when coincubated in shaken culture. Under these conditions, tight particle adhesion is a prerequisite for phagocytosis, and mutants have been isolated which

are able to phagocytose when plated on a bacterial lawn but not in suspension (Ceccarelli and Bozzaro, 1992; Cohen *et al.*, 1994; Cornillon *et al.*, 2000). In principle, these mutants could be defective in membrane receptors that mediate particle binding, in actin cytoskeletal components that stabilize adhesion and give rise to the phagocytic cup, or signaling factors that regulate binding and/or actin rearrangement.

Such a well-defined mutant is a talin-null mutant, which is strongly defective in both phagocytosis of *Escherichia coli* B/r in suspension and cell adhesion to plastic or glass. Only bacteria devoid of the carbohydrate moiety of cell surface lipolysaccharides, such as the rough *Salmonella minnesota* R595 strain, are adhesive enough to be recruited by talin-null cells and phagocytosed (Niewohner *et al.*, 1997). The talin-null mutant illustrates the complexity of the potential receptor–cytoskeletal interactions in phagocytosis. Talin is known to mediate F-actin interaction with the membrane, though it is not essential in *Dictyostelium*, as macropinocytosis is not affected in the mutant (Niewohner *et al.*, 1997). Bacterial binding, in contrast, has to be stable enough for the particle to be engulfed. It has been suggested that talin could be part of a specific complex with binding receptors for *E. coli*, not however for *S. minnesota* R595. Weakening of this complex would then reduce selectively *E. coli* uptake in the mutant. Alternatively, *S. minnesota* R595 binding may be stronger, despite talin absence, thus resisting the shear forces generated by shaking and ultimately leading to uptake (Niewohner *et al.*, 1997).

The search for membrane receptors involved in phagocytosis has been elusive in *Dictyostelium* for several years, though there is some indication that things may now change. The existence of different receptors for bacteria, latex beads or yeast particles was first inferred by Hellio and Ryter (1980) in inhibition experiments with lectins. Yeast uptake, in particular, correlated with Wheat Germ Agglutinin-binding membrane receptors. By using chemical mutagenesis, Vogel and his coworkers (1980) provided evidence for the existence of two types of receptors on the surface of *Dictyostelium* cells for *E. coli* B/r. A mutant was isolated that failed to ingest hydrophilic latex beads, while retaining the ability to phagocytose bacteria. Phagocytosis in the mutant, but not in the parental strain, could be inhibited by the addition of glucose, suggesting the existence of two receptors; a lectin-type receptor that could interact with the terminal glucose on the *E. coli* lipopolysaccharide, and a ''nonspecific'' receptor, inactive in the mutant and responsible for the uptake of hydrophilic latex beads. Shortly later, using sugar-derivatized polyacrylamide flat gels, it was shown that *Dictyostelium* cells possessed three surface receptors for binding to glucosides, mannosides, or *N*-acetylglucosaminides (Bozzaro and Roseman, 1983a). Binding to glucoside was particularly interesting, as it interfered in a subtle way with the ability of the cells to form stable aggregates, leading to the suggestion that the glucoside receptor could act as a sensor for bacteria (Bozzaro and

Roseman, 1983b; Gambino *et al.*, 1992). Chemical mutagenesis and selection for binding to sugar-derivatized gels resulted in mutants that failed to bind to glucosides. The mutants displayed strongly reduced phagocytosis of *E. coli* B/r, but not latex beads (Ceccarelli and Bozzaro, 1992), thus confirming Vogel's hypothesis of two distinct membrane receptors. Unfortunately, chemical mutagenesis in *Dictyostelium* does not allow identifying the mutated genes, and biochemical attempts to purify the potential receptors have been unsuccessful.

The approach of Vogel and coworkers has been recently combined with restriction enzyme-mediated insertion transformation to generate randomly mutagenized tagged cell populations and select for phagocytosis mutants (Cornillon *et al.*, 2002). Two mutants were isolated that were defective for phagocytosis of latex beads, but not bacteria (*Klebsiella pneumoniae* or *E. coli*). The mutants displayed also reduced substrate adhesion and motility, suggesting a basic common role for the tagged genes. The candidate genes, Phg1 and Phg2, belong to a family of nine-transmembrane domain proteins present in plants, yeast and humans, but fail to meet the criteria of adhesion proteins. Phg1 definitely does not act as phagocytosis receptor, rather it has been proposed to regulate phagocytosis, possibly by modulating surface expression of other proteins involved in adhesion (Benghezal *et al.*, 2003). Phg2 has been recently shown to be a serine/threonine kinase that regulates actin cytoskeleton dynamics, adhesion, and motility, in addition to phagocytosis (Blanc *et al.*, 2005) (see Section 4.2).

Another early candidate, gp130, also fails to meet the criteria of phagocytic receptor. Gp130 was identified as potential target of a poly-specific antiserum that inhibited phagocytosis as well as the EDTA-labile form of intercellular adhesion (Chia and Luna, 1989). The notion that a common receptor may be involved in phagocytosis and EDTA-labile cell–cell adhesion is appealing, as the same receptor may be used to sense the environment, allowing preaggregative starving cells to rapidly switch from development to growth, if bacteria turn to be available. However, a gp130 knockout mutant failed to display any defect in both functions (Chia *et al.*, 2005). In addition, the most prominent candidate for EDTA-labile cell–cell adhesion, the 27 glycoprotein DdcadA, belonging to the cadherin family, is not required for phagocytosis (Chia *et al.*, 2005). A promising candidate gene in this context is *sadA*, which encodes an integral membrane protein, and whose deletion results in cells strongly defective in binding to glass substratum, in phagocytosis and EDTA-labile contacts (Fey *et al.*, 2002). Functional studies are required to better define SadA role in phagocytosis.

A family of five proteins, distantly related to human β-integrin, have been recently identified (Cornillon *et al.*, 2006). These proteins, named Sib (similar to integrin beta)-A to -E, display a conserved cytoplasmic region that binds the cytoskeletal protein talin. Disruption of SibA results in cells defective in latex beads, but not bacteria (*Klebsiella*), phagocytosis. The cells

are also defective in cell-substratum, though not in cell–cell adhesion (Cornillon *et al.*, 2006). SibA could thus be one of nonspecific ''hydrophilic'' receptors hypothesized by Vogel *et al.* (1980).

Genes encoding potential phagocytosis receptors have been detected in a recent genome-wide transcriptomic study designed to identify genes relevant for phagocytosis of or growth on *E. coli* (Sillo *et al.*, 2008). Transcripts from cells exposed briefly to the bacteria or in exponential growth on bacteria were compared with each other and with transcripts from axenically growing cells. Clusters of genes could be identified that were regulated by either phagocytosis or growth on bacteria. Among genes strongly upregulated by phagocytosis are genes encoding a carbohydrate-binding membrane protein, a putative scavenger receptor, and a few other putative membrane proteins. The potential role of these genes in phagocytosis can now be analyzed by generating knockout mutants and by functional studies.

3.2. The actin cytoskeleton in phagocytosis

The colocalization studies of actin and actin-binding proteins with the phagocytic cup and the phagosome, mentioned in the first section, pinpoint the importance of the actin cytoskeleton in the process. Phagocytosis is inhibited by drugs affecting actin polymerization such as cytochalasin or latrunculin (Hacker *et al.*, 1997; Maniak *et al.*, 1995). No similar reports exist on drugs affecting the microtubule cytoskeleton, but microtubules have been suggested to be involved in intracellular transport of phagosomes (Clarke and Maddera, 2006).

The *Dictyostelium* genome harbours members of the two major actin-nucleating families, the Arp2/3 complex and formin, as well as their regulators, Scar/Wave, WASP, and ENA-VASP. Scar-Wave, WASP, or ENA-VASP are responsible for the localized activation of Arp2/3 or formin in the membrane, in response to signals mediated by small G proteins and phosphoinositides. The Arp2/3 complex induces actin nucleation into a filament network, while formin is mainly responsible for actin filament elongation (Faix and Grosse, 2006; Ibarra *et al.*, 2005). Schematically, formin is responsible for membrane structures such as filopodia, whereas the Arp2/3 complex induces pseudopodial and lamellipodial extensions. Arp2/3 and Scar/WAVE are recruited within seconds to phagocytic or macropinocytic cups, concomitantly with actin polymerization (Insall *et al.*, 2001). Disruption of the single *scar/Wave* gene leads to a strong decrease in the rate of phagocytosis and macropinocytosis, concomitant with a 50% decrease in F-actin cell content (Seastone *et al.*, 2001). There are two WASP encoding genes in *Dictyostelium* and the double knockout is probably lethal. Mutants having WASP1 disrupted and WASP2 knocked down display reduced chemotaxis. Whether they are also impaired in phagocytosis

has not been reported (Myers *et al.*, 2005). Formin, which is essential for filopodia formation, is also found beneath the phagocytic cup (Faix, personal communication), but its disruption, as well as inactivation of the regulatory *Vasp* gene, while affecting particle adhesion, does not hinder phagocytosis (Han *et al.*, 2002).

In addition to F-actin-nucleating factors, remodeling of the actin cortex requires the activity of a variety of actin-binding proteins, which regulate either availability of actin monomers, bundling, cross-linking, or fragmenting of actin filaments or their association with the plasma membrane. Because of the prominent role of motility processes in *Dictyostelium* development, several studies have been performed to examine the function of these proteins (reviewed in Noegel and Schleicher, 2000). Several actin-binding proteins are transiently recruited to the phagocytic cup and/or the phagosome, with kinetics roughly similar to actin itself, but only a few of them have been shown, by gene knockout or overexpression experiments, to control phagocytosis. Negative results may be due, in some cases, to redundancy in the cytoskeletal proteins, which can be eventually bypassed by generating double or triple mutants. Thus, single mutants for the F-actin cross-linking proteins α-actinin or gelation factor are mildly defective in phagocytosis, but a double mutant displays a 50% reduction in the phagocytosis rate (Rivero *et al.*, 1996). A profilin I/II double mutant, in contrast to the single mutants, is characterized by enhanced phagocytic activity and a thick actin cortex, due to higher F-actin content (Temesvari *et al.*, 2000). The thick actin cortex favors in some way phagocytosis. The mutant phenotype can be reversed by second suppression of the CD36/LIMPII homologue, LimpA, a protein that is located on endolysosomal vesicles. The interaction between these two genes is unclear, except that both profilins and LimpA bind to PI(4,5)P2. It has been reported that LimpA inactivation reduces the abnormal F-actin content in the double mutant, and this could be the immediate reason for the rescuing effect on phagocytosis (Temesvari *et al.*, 2000).

The F-actin destabilizing protein cofilin, and its regulator protein Aip1, localize to phagocytic cups and stimulate membrane ruffles (Aizawa *et al.*, 1997; Konzok et al, 1999). Cofilin disruption is very likely lethal (Aizawa *et al.*, 1996), but Aip1 disruption impairs phagocytosis, while overexpression stimulates it (Konzok *et al.*, 1999). The reduction in the phagocytosis rate in the Aip1 null mutant has been correlated with a slower rate of membrane protrusion around the phagocytic cup, suggesting that Aip1 enhances *via* cofilin the pool of actin monomers available for new actin filaments at the phagocytic rim (Konzok *et al.*, 1999). Cofilin overexpression, however, has no effect on phagocytosis (Aizawa *et al.*, 1996). Coronin, an actin-associated protein found in many species, was originally shown in *Dictyostelium* to be enriched at the leading front of chemotaxing cells and in crown-shaped extensions of axenically growing cells (de Hostos *et al.*, 1991).

The protein is also transiently recruited to phagocytic cup and is released from the phagosome shortly after its closure. Knockout mutants are strongly defective in yeast particle uptake (Maniak *et al.*, 1995).

Early phagosomes are also coated with comitin, an F-actin-binding protein that is associated with Golgi and post-Golgi vesicle membranes. Comitin knockout mutants are defective in phagocytosis of yeast particles and *E. coli*, but not latex beads. The mutants are otherwise normal for growth, pinocytosis, secretion, chemotaxis, and motility (Schreiner *et al.*, 2002). The reduced uptake of *E. coli*, in contrast to latex beads, suggests that the defect may be linked with particle binding, rather than uptake, considering that both particles are of similar size but the *E. coli* surface is more hydrophilic. Comitin would then resemble talin in its effects, though the mechanism of action remains obscure.

Myosins serve as actin-linked motors and are expected to play a major role in regulating contraction, intracellular vesicle delivery, and thus migration, cytokinesis, maintenance of cell shape, morphogenesis, and least, but not last, phagocytosis (Yumura and Uyeda, 2003). Class I and VII myosins have been shown to be required for phagocytosis in *Dictyostelium*, whereas the conventional myosin II is not essential (Durrwang *et al.*, 2006; Fukui *et al.*, 1990; Maselli *et al.*, 2002; Titus, 1999). Disruption of myosin VII, in particular, results in strong defect in initial particle binding, and thus in particle uptake (Titus, 1999). Intriguingly, MyoVII forms a complex with talin, and its disruption leads to an 80% decrease in cytosolic talin content, suggesting that the strong defect in the myoVII mutant may be indirectly mediated by depletion of talin (Galdeen *et al.*, 2007). Knocking out MyoIK, MyoIA/IB, IA/IC, or MyoIE also affects phagocytosis, mainly due to reduced cortical tension and particle binding (Durrwang *et al.*, 2006; Jung *et al.*, 1996; Schwarz *et al.*, 2000; Soldati, 2003). It is likely that the major function of these unconventional myosins is to deliver components that are required for membrane recycling and extension rather than providing the force for internalization. This would fit with the observations that myosin VII is found at filopodial tips, but not in phagocytic cups, and that other class I myosins reside only transiently in phagocytic cups or phagosomes (Durrwang *et al.*, 2006; Schwarz *et al.*, 2000; Tuxworth *et al.*, 2001).

Actomyosin interactions are regulated by myosin heavy chain (MHC) and light chain (MLC) kinases. PakB, the myosin I heavy chain kinase, when expressed in the activated form, is strongly enriched in macropinocytic and phagocytic cups, and leads to increased phagocytosis (de la Roche *et al.*, 2005). Remarkably, MHCK (myosin II heavy chain kinase), whose activity leads to myosin II disassembly from the actin cytoskeleton, is localized to phagocytic cups (Steimle *et al.*, 2001). MHCK activity could be responsible for myosin II exclusion from the phagocytic cup, thus locally inhibiting actomyosin contractile fiber formation and rather favoring actin filament assembly into pseudopodial-like extensions.

3.3. Phagosome fusion with endolysosomal vesicles and killing of bacteria: The other players

The ultimate outcome of phagocytosis is killing and digestion of the internalized bacteria. This is achieved by activation of several processes: production of toxic oxygen radicals (respiratory burst), acidification of the phagosomal lumen and depletion of essential divalent metals, degradation of the bacterial cell wall, and digestion by hydrolytic enzymes.

In phagocytic leukocytes, a membrane-associated NADPH oxidase complex produces large quantities of superoxide radicals from molecular oxygen. Dismutation of O_2^- to H_2O_2 and formation of hydroxyl radicals (OH^-) and hyperchlorous acid (HOCl) generate potent microbicidal compounds (Minakami and Sumimotoa, 2006). The NADPH oxidase (cytochrome b558) is made of at least two membrane-bound subunits and two cytosolic components, is usually inactive, and is activated by a variety of stimuli associated with phagocytosis. The cytosolic components are targeted to phagosomes in a process involving the small G proteins Rac1 or Rac2, which activate the oxidase (Minakami and Sumimotoa, 2006). *Dictyostelium* cells possess three isoforms (noxA, noxB, noxC) of the large cytochrome b558 subunit and an isoform ($p22^{phox}$) of the small subunit. A double knockout $noxA^-/noxB^-$ displays normal phagocytosis and macropinocytosis. More importantly, no evidence has been provided so far for superoxide production in *Dictyostelium in vivo* or *in vitro* (Lardy *et al.*, 2005). Thus, whether a respiratory burst is induced during phagocytosis in *Dictyostelium* remains open.

As mentioned in Section 2, the phagosome undergoes extensive remodeling in its route to phagolysosomal maturation. The phagosomal vesicle looses quite rapidly its actin coat and undergoes rapid acidification (Aubry *et al.*, 1993; Padh *et al.*, 1993). The kinetics of uncoating is roughly similar for actin (Peracino *et al.*, 1998), actin-binding proteins (Insall *et al.*, 2001; Konzok *et al.*, 1999; Lee *et al.*, 2001; Maniak *et al.*, 1995; Rupper *et al.*, 2001b), as well as signal transducers and effectors, such as RacF1 (Rivero *et al.*, 1999), the myosin I heavy chain kinase PakB (de la Roche *et al.*, 2005), or myosin II heavy chain kinase (Gotthardt *et al.*, 2002; Steimle *et al.*, 2001). The V-H^+ ATPase, the major if not unique agent of acidification, is enriched in the contractile vacuole (CV) system and in acidic vesicles independent of the CV. The latter vesicles fuse indifferently with phagosomes and macropinosomes, and are considered a prelysosomal vacuolar reservoir (Clarke *et al.*, 2002a,b; Neuhaus *et al.*, 2002; Padh *et al.*, 1989; Peracino *et al.*, 2006; Souza *et al.*, 1997).

Prelysosomal nonacidic vesicles have also been described, which contain on their surface Natural Resistance Associated Membrane Protein (Nramp)1, an endolysosomal divalent metal transporter conferring resistance to invasive pathogenic bacteria, as will be discussed in Section 5.2. These vesicles

are distributed in the cytoplasm, enriched in the trans-Golgi network, where they colocalize with comitin and the Vti1 SNARE protein, and undergo rapid fusion with phagosomes or macropinosomes, immediately after their acidification (Peracino *et al.*, 2006). Fusion of Nramp1-decorated vesicles with phagosomes is very likely stimulated by Vt1, since this protein colocalizes with Nramp1-decorated vesicles also outside of the Golgi. As mentioned in Section 2, the Vti1 protein is part of a SNARE complex with syntaxin 7, syntaxin 8, and VAMP7 in early endosomes and mediates endosome fusion with lysosomal vesicles (Bogdanovic *et al.*, 2002). Disruption of the Nramp1 encoding gene has only mild effects on phagocytosis and growth on *E. coli*, suggesting that the Nramp1 function, namely depleting the phagosome of divalent metals, in particular iron, is not essential for killing nonpathogenic bacteria (Peracino *et al.*, 2006).

Another protein, which is required for delivery of both V-H^+-ATPase- and Nramp1-decorated vesicles to phagosomes, is the actin-binding, cyclase-associated protein (CAP) (Sultana *et al.*, 2005). CAP binds the vatB subunit of the vacuolar ATPase and is supposed to mediate its interaction with the actin cytoskeleton. CAP-null mutants display altered distribution of both V-H^+-ATPase- and Nramp1-decorated vesicles and higher endosomal pH (Sultana *et al.*, 2005). Digestion of bacteria is accomplished by different types of lysosomal hydrolases, which are abundant in vegetative *Dictyostelium* cells, and are delivered in successive phases to phagosomes (Gotthardt *et al.*, 2002; Rodriguez-Paris *et al.*, 1993; Souza *et al.*, 1997; Temesvari *et al.*, 1994).

The *Dictyostelium* genome also encodes a large family of pore-forming peptides, which act by perforating the membrane of bacteria. Some are synthesized as prepropeptide precursors, like the mammalian defensins, others as prepromultipeptide precursors, like the naegleriapores (Leippe *et al.*, 2005), and may give rise to a large variety of peptides upon hydrolytic cleavage. Two of them have been shown to be potent antibacterial agents *in vitro*, and have been proposed to kill the bacteria in the phagosome, before these are degraded by hydrolases (Leippe, personal communication). The *Dictyostelium* genome harbours several lysozyme-encoding genes, some belonging to the bacteriophage T4 type and others to the C-type lysozymes. A novel unconventional class, the ALY class, including four members, has been described. The purified ALYA lysozyme displays antibacterial activity against Gram-positive, but not Gram-negative bacteria. Disruption of the *alyA* gene results in a 60% reduction of total lysozyme activity, and does not hinder growth on *E. coli*, though increased phagocytosis has been reported, possibly to compensate for defective digestion (Mueller *et al.*, 2005).

The postlysosomal vesicle, devoid of the vacuolar ATPase, is coated with coronin, actin, and vacuolin A and B. Both vacuolin A and B are postlysosomal markers (Rauchenberger *et al.*, 1997). Vesicles decorated with both Nramp1 and vacuolin coexist with vesicles decorated with

vacuolin only. It is possible that Nramp1- and vacuolin-positive vesicles represent an intermediate step in the postlysosomal pathway (Peracino *et al.*, 2006). Disruption of the vacuolin A encoding gene has no effects. A vacuolin B-null mutant, instead, displays abnormally large postlysosomal vacuoles and delayed release of fluid-phase marker, though surprisingly these defects do not affect the rate of cell growth in axenic medium or bacteria (Jenne *et al.*, 1998). Maturation of postlysosomal vesicles is also impaired in the LvsB-null mutant (Charette and Cosson, 2007; Harris *et al.*, 2002). LvsB is a protein most similar to LYST/Beige, the gene that is mutated in the Chediak-Higashi Syndrome, which is characterized by the presence of enlarged lysosomes. In the LvsB-null mutant, there is abnormal acidification of postlysosomal compartments, probably due to inappropriate fusion of early endosomes and postlysosomal vesicles (Kypri *et al.*, 2007), supporting the notion that LvsB is a negative regulator of heterotypic fusion (Harris *et al.*, 2002).

4. Regulatory Pathways Controlling Phagocytosis

Phagocytic cup formation and closure, as well as postlysosomal vesicle exocytosis are mainly actin-cytoskeleton-based processes. It is therefore not surprising that signal transduction pathways regulating the actin cortex also regulate phagocytosis. Assembly and reshaping of the actin meshwork are controlled by signals originating at the site of particle attachment and transmitted to the cell interior by heterotrimeric G proteins, monomeric G proteins of the Ras and Rac family, and by enzymes regulating membrane phospholipids, in particular phosphoinositides. Cytosolic tyrosine kinases, particularly of the *syk* family, are important effectors in macrophage FcγR-dependent phagocytosis (Greenberg, 1995). A similar role for *Dictyostelium* tyrosine kinases has not been reported, though a membrane tyrosine kinase has been recently involved in phagosomal maturation (see further below). Phagosomal traffic, like all intracellular traffic, is also controlled by the small G proteins of the Rab family (Zerial and McBride, 2001), which is largely represented in the *Dictyostelium* genome.

4.1. Heterotrimeric G protein in phagocytosis

The heterotrimeric G protein is a major regulator of chemotaxis-based motility both during growth and development of *Dictyostelium*. It is activated by canonical seven-transmembrane receptors and, in addition to chemotaxis, regulates also pathways controlling cell development and differentiation (Manahan *et al.*, 2004). Several Gα subunits exist in

Dictyostelium, which are expressed at different times during growth or development and associate with one Gβ and one Gγ subunit (Wu and Devreotes, 1991). Indirect evidence for G protein involvement in phagocytosis was provided by using aluminum fluoride as inhibitor (Browning and O'Day, 1995), whereas direct evidence was obtained by knocking out the Gβ subunit. The G$\tilde{\beta}$-null mutant displays strong reduced phagocytosis rate of bacteria, and to a lower extent latex beads, but is normal for macropinocytosis, thus providing a first evidence for signaling discriminating phagocytosis from macropinocytosis (Peracino *et al.*, 1998). The phagocytosis defect in the mutant correlates with inefficient actin reorganization at the rim of the progressing phagocytic cup, and thus with phagosome closure, while particle binding as well as initial phagocytic cup formation at the site of adhesion are almost unaffected. This raises the possibility that actin recruitment at the site of particle binding is a stochastic process, independent of signaling. Indeed, intense and spontaneous actin remodeling in the absence of signals occurs incessantly in the actin cortex in the order of subseconds (Diez *et al.*, 2005). This intense activity has been proposed to confer the plasticity required for rapidly forming phagocytic cups and adapting to external stimuli (Diez *et al.*, 2005), but it can also explain why many phagocytic events are aborted also in wild-type cells. If so, then the major effect of heterotrimeric G protein activation, following particle binding, could be to superimpose local organization to a stochastic process.

A possible Gα partner of the Gβ subunit is Gα4. This alpha subunit is enriched in purified phagosomes together with the Gβ at earliest time points, coinciding with phagosome formation, and at late maturation stages, close to exocytosis (Gotthardt *et al.*, 2006). Since the postlysosomal vesicles are recoated with actin before excretion, these data suggest a close link between the heterotrimeric G protein and actin reorganization at different steps of the phagocytic process. As expected if Gα4 is the relevant Gα subunit associated to Gβ, Gα4 gene inactivation also results in reduced phagocytosis rate (Gotthardt *et al.*, 2006).

How the heterotrimeric G protein is activated remains open. A possibility is lateral clustering of particle-binding membrane proteins with seven-transmembrane receptors to form a signaling complex that may activate the G protein. A recent DNA microarray study has shown that two of the five genes encoding tetraspanins in *Dictyostelium* genome are upregulated in cells undergoing phagocytosis (Sillo *et al.*, 2008). Tetraspanins are four-transmembrane proteins, which act as scaffold to link together membrane receptors and other signaling effectors (Hemler, 2005). Tetraspanins have been recently involved in integrin-dependent phagocytosis in retinal pigment epithelia and in antigen-presenting cells (Chang and Finnemann, 2007), and tetraspanin-clustering with a G protein-coupled receptor (GPCR) has been also reported (Little *et al.*, 2004). Tetraspanins may thus furnish the link for clustering particle-binding membrane proteins with

GPCR's into a signaling complex, which regulates actin recruitment and, possibly, membrane recycling and gene expression during phagocytosis. The Gβ protein may act as integrator of signals arising in this signaling complex, independently of a specific ligand binding to a GPCR. Alternatively, particle binding may stimulate local secretion of cytokines that in turn activate GPCR's and thus the hetrotrimeric G protein. Folate and other secreted bacterial metabolites are known to act as chemoattractants in *Dictyostelium*. Since the Gβ-null mutant is, however, also partially defective in latex beads uptake, an autocrine cytokine would be a more appealing candidate. Such a potential candidate is GABA (γ-aminobutyric acid), for which G protein linked $GABA_B$-like receptors (GPCR family 3) exist in *Dictyostelium* (Prabhu and Eichinger, 2006). Genes encoding two such receptors have been shown to be upregulated by phagocytosis (Sillo *et al.*, 2008). In addition, phagocytosis leads to coregulation of genes involved in glutamate and GABA biosynthesis (Sillo *et al.*, 2008). Functional studies by generating null mutants for genes encoding tetraspanin or glutamate decarboxylase can validate these hypotheses.

4.2. Phosphoinositides and calcium ions

Candidate downstream transducers of the heterotrimeric G protein and/or particle-binding membrane receptors are small G proteins of the Ras and Rac families, membrane lipids generated by the activity of phospholipases, PI kinases and phosphatases, and calcium ions.

Phosphatidylinositide metabolism, in particular, plays a major role in signal transduction, actin remodeling, and membrane trafficking in eukaryotic cells, including *Dictyostelium* (Payrastre *et al.*, 2001; Willard and Devreotes, 2006; Yeung and Grinstein, 2007). Phosphatidylinositides are glycerolipids containing a D-*myo*-inositol head group, which can be phosphorylated at positions 3, 4, and 5 by specific PI kinases. In addition to PI kinases, activation of phospoholipase C or PI phosphatases generate different classes of lipid species, which act as binding sites for effector proteins with specific recognition domains, such as PH, PX, FYVE, ENTH/ANTH, and FERM domains (Downes *et al.*, 2005; Lemmon, 2003, 2007).

Phosphoinositides account for 11% and 9% of total lipids in *Dictyostelium* plasma membrane and endolysosomal membranes, respectively (Nolta *et al.*, 1991). Phosphoinositide dynamics during particle uptake in *Dictyostelium* has been studied by using GFP-fused PH domains with different PI specificities (Dormann *et al.*, 2004; Blanc *et al.*, 2005; Loovers *et al.*, 2007), and can be summarized as follows. PI(4,5)P2 (phosphatidylinositol, 4–5, bisphosphate) is distributed uniformly on the plasma membrane, and may be responsible for basal actin enrichment in the membrane cortex. Although the details remain controversial, it is established that PI(4,5)P2 is important in both activation and localization of actin nucleation factors and actin-binding

proteins, such as profilin, coronin, gelsolin, DAPI1, or CAP. It is unclear whether there is a burst of PI(4,5)P2 accumulation in the phagocytic cup, but PI(4,5)P2 declines concomitantly with closure of the phagocytic cup, starting at its base, while PI(3,4,5)P3 accumulates and peaks just after phagosome closure, disappearing rapidly thereafter. PI(3,4)P2 increases concomitantly with PI(3,4,5)P3, but declines with a much slower rate. Disassembly of the actin coat from the internalized phagosome correlates timely with PI(4,5)P2 decrease, rather than with disappearance of PI(3,4,5)P3 or PI(3,4)P2 (Fig. 6.2).

PI(4,5)P2 is the target of PLC or PI3K activity, yielding the second messengers diacylglycerol (DAG) and inositol(1,4,5)P3 (IP3), or PI(3,4,5)P3, respectively (Fig. 6.3). A pharmacological approach to characterize signal transduction pathways regulating phagocytosis has led to identical results in two different labs (Peracino *et al.*, 1998; Seastone *et al.*, 1999), namely the requirement of phospholipase C (PLC) and intracellular calcium for efficient phagocytosis. PLC inhibitors and intracellular calcium chelators inhibited significantly the extent of phagocytosis, whereas PKA, PKC, PLA2, or tyrosine kinase inhibitors as well as PI3K inhibitors did not affect bacterial uptake. Remarkably, PI3K inhibitors affect macropinocytosis (Seastone *et al.*, 1999). In addition to pharmacological data, several lines of evidence support a central role for PI(4,5)P2 and PLC activity in phagocytosis. The bulk of PI(4,5)P2 is reduced to 40% of control in a *myo*-inositol auxotrophic mutant following *myo*-inositol starvation. Under these

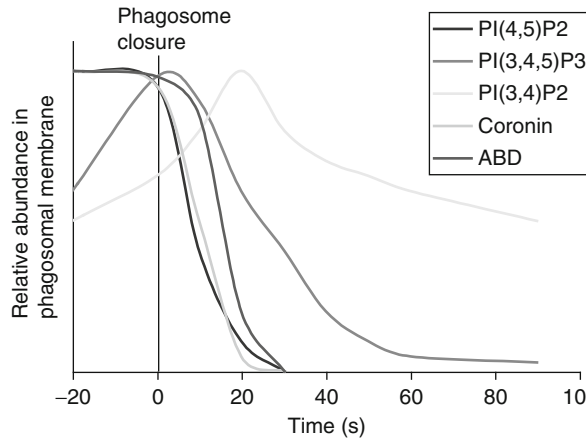

Figure 6.2 Time course of phosphoinositide metabolism and recruitment of actin-binding proteins during phagocytosis. The relative abundance of different phosphoinositide types in the membrane of phagocytic cup/phagosome was measured over time by using GFP fused to specific PH domains. Relative changes in the binding of GFP-coronin and GFP-ABD probes to phagocytic cup/phagosome are also shown. The figure summarizes data from the following references: Dormann *et al.* (2004), Blanc *et al.* (2005), Loovers *et al.* (2007).

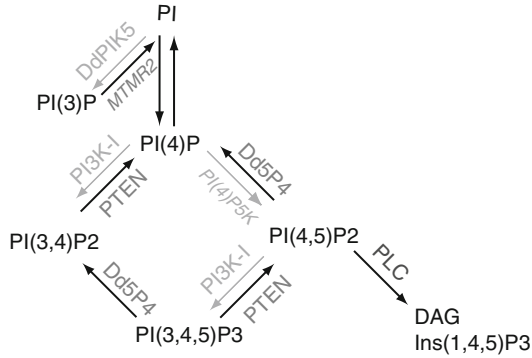

Figure 6.3 Pathways for interconversion of phosphatidylinositol polyphosphates involved in *Dictyostelium* phagocytosis. Only identified gene products, and their pathways, are shown. Phosphorylation reactions and kinases are in light grey; dephosphorylation reactions and phosphatases are in dark grey. Phospholipase C (PLC) is in black. In bold are enzymes, whose encoding genes have been disrupted and/or the activity studied with specific inhibitors; in italics are indicated gene products, which have been identified, but their activity in phagocytosis has not been studied so far. Abbreviations: PI, phosphatidylinositol; PI(X)P, phosphatidylinositol(X)phosphate; DAG, diacylglcyerol; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; PTEN, phosphatase and tensin homologue (encoded by 1 gene); PI3K-I, phosphatidylinositol 3-kinase, class I (encoded by 3 genes); MTMR2 (myotubularin related phosphatase 2); Dd5P4, OCRL-1 homologue inositol 5-phosphatase; DdPIK5, PI3 kinase specific for PI. See text for details.

conditions, bacterial uptake, but not fluid-phase endocytosis, is strongly reduced in the mutant (Fischbach *et al.*, 2006).

Particle binding and phagocytic cup formation are inhibited in the Phg2-null mutant defective in a serine/threonine kinase that is associated to the plasma membrane. This association is mediated by a PH domain that binds to PI(4,5)P2 or PI(4)P sites. Deletion of this binding region in the Phg2 kinase prevents complementation of the phagocytic defect in the null mutant (Blanc *et al.*, 2005). As already mentioned, the Phg2-null mutant is defective in phagocytosis, but not in macropinocytosis. Whatever the function of this kinase in phagocytosis, the finding that its activity requires binding to PI(4,5)P2 or PI(4)P sites supports the idea that these PI sites are important for phagosome formation.

This conclusion is further supported by another study, in which phospholipase D (PLD) activity was inhibited with butan-1-ol (Zouwail *et al.*, 2005). Inhibition resulted in strong reduction of *de novo* synthesis of PI(4,5)P2, probably due to PLD-dependent stimulation of a PI(4)P5K activity acting on PI(4)P (Fig. 6.3). PLD is supposed to activate PI(4)P5K via production of the second messenger phosphatidic acid. Phagocytosis was partially inhibited in cells treated with butan-1-ol. F-actin recruitment to membrane extensions was also inhibited, with retraction of filopodia and pseudopodia, while most F-actin localized in intracellular spots. The bulk of

PI(4,5)P2 is presumably not affected by the short treatment with butan-1-ol, rather fluctuations in PI(4,5)P2 concentrations, regulated by PLD, have been suggested to be important for controlling local actin recruitment (Zouwail *et al.*, 2005). Interestingly, ABD-GFP localized to the cell cortex, suggesting that the PI(3,4,5)P3 binding sites were mostly unaltered upon butan-1-ol treatment.

These data support a model whereby biosynthesis or hydrolysis of PI(4,5)P2 regulate actin recruitment to the phagocytic cup and actin disassembly from the phagosome, respectively. Inhibiting PLC may result in inefficient disassembly of the actin coat, and thus inhibition of phagosomal membrane fusion and/or phagosome detachment from the membrane. Studies in mammalian cells have shown that PI(4,5)P2 sites are effectively removed from the plasma membrane by PLC activation, but not by PI3K (Stauffer *et al.*, 1998). In *Dictyostelium*, the PTEN 3-phosphatase is associated with the membrane and its removal is a prerequisite for accumulation of PI(3,4,5)P3, independently of PI3K recruitment to the membrane (Iijima and Devreotes, 2002). PTEN binds to PI(4,5)P2 sites, thus it can be hypothesized that PLC activation leads to PTEN removal, and PTEN release in turn favors formation of PI(3,4,5)P3 by PI3K acting on the remaining PI(4,5)P2 sites. Indeed, PTEN is removed quite rapidly and selectively from the base of the progressing phagocytic cup (Dormann *et al.*, 2004). PI3K could thus contribute to actin disassembly from the phagosome, but PI(4,5)P2 hydrolysis by PLC activity appears to be the most relevant factor, which would be consistent with the dynamics of PI sites and actin disassembly shown in Fig. 6.2 as well as the differential results with PLC and PI3K inhibitors on phagocytosis mentioned above.

Dictyostelium PLC is similar to mammalian PLC-δ, and is activated by calcium, like the mammalian homologue (Drayer and van Haastert, 1992). As mentioned, intracellular calcium chelators also inhibit phagocytosis (Peracino *et al.*, 1998; Seastone *et al.*, 1999). Calcium is stored in the endoplasmic reticulum (ER), and tubules of the ER have been shown to come into close association with phagosomes during uptake, though no fusion of ER membranes with the phagosome has been observed (Lu and Clarke, 2005; Muller-Taubenberger *et al.*, 2001). It is possible that association of ER with the phagocytic cup is required for local release of calcium, following phagocytic stimuli. Calcium increase may stimulate PLC activity as well as activate proteins involved in phagosomal membrane tethering and fusion.

In addition to the pharmacological approach with inhibitors, the involvement of PI3K and the antagonist PTEN phosphatase in phagocytosis has been studied with mutants. Disrupting the single *pten* gene has no effect on bacteria phagocytosis, but reduces yeast particle uptake (Dormann *et al.*, 2004). There are six PI3K genes in the *Dictyostelium* genome, five belonging to the class IB group and one to class III. The class IB enzymes consist of the p110γ catalitic subunit, and a regulatory subunit, the p101 protein. Double

disruption of PI3K1/2 has no effect on bacteria and latex beads phagocytosis rate, but inhibits endocytosis (Buczynski *et al.*, 1997b; Zhou *et al.*, 1998), thus confirming the data with PI3K inhibitors. The double mutant is characterized by strongly reduced levels of both PI(3,4)P2 and PI(3,4,5)P3 as well as strong reduction of F-actin-enriched ruffles and crown-shaped membrane extensions, which are typical of pinocytosing cells (Zhou *et al.*, 1998). The observation that this clear-cut effect on the actin cortex does not inhibit bacterial phagocytosis, though inhibiting macropinocytosis, suggests that the latter process is more sensitive than phagocytosis to changes in actin dynamics. This is in agreement with recent findings from DNA microarray studies, indicating that genes encoding actins, several actin-binding proteins and myosins are upregulated in cells undergoing macropinocytosis compared to phagocytosis (Sillo *et al.*, 2008).

A differential requirement for PI3K in phagocytosis is suggested by the work of Dormann *et al.* (2004). These authors found that PI3K inhibitors inhibited bacterial uptake, in contrast to previous reports. However, the assay used to measure uptake, namely a decrease in the optical density of a bacterial suspension over 7 h, does not allow distinguishing effects of the inhibitor on bacterial uptake from effects on phagolysosome maturation and bacterial degradation. In contrast, the assay used for yeast particle uptake in the same paper was a short-term assay and inhibition of uptake was evident. It is therefore possible that there is a differential requirement for PI3K, depending on the particle to be internalized. This conclusion is further supported by the finding that PTEN disruption has no effect on bacterial, but a partial inhibitory effect on yeast uptake (Dormann *et al.*, 2004). It is conceivable that the combined activity of PLC and PI3K leads to a more rapid disappearance of PI(4,5)P2 sites, and this effect may be more relevant for phagocytosis of larger rather than smaller particles.

In addition to PLC and PI3K, the product of the OCRL-1 homologue Dd5P4 gene, the inositol 5-phosphatase, also tags PI(4,5)P2, catalyzing its dephosphorylation into PI(4)P (Loovers *et al.*, 2007). This phosphatase can also dephosphorylates PI(3,4,5)P3 (Fig. 6.3). Dd5P4-null mutants are defective in both yeast particle phagocytosis and macropinocytosis (Loovers *et al.*, 2007). The defect in phagocytosis has been related to impaired progression and closure of the phagocytic cup, rather than particle binding or phagocytic cup formation. It has been proposed that lack of conversion of PI(3,4,5)P3 to PI(3,4)P2 may be responsible for this inhibition. However, the Dd5P4 phosphatase displays higher specificity for PI(4,5)P2 than PI (3,4,5)P3 (Fischbach *et al.*, 2006), therefore other possible explanations are at hand: increased PI(4,5)P2 expression in the mutant, as a result of Dd5P4 gene disruption, would be consistent with the observed inhibition of phagocytosis as well as with the above-mentioned PLC inhibition data. PI (4,5)P2 persistence would lead, in both cases, to delayed actin disassembly and thus to defects in phagosome closure and further maturation. This idea

fits also well with the finding that total PI(3,4)P2 and PI(3,4,5)P3 sites were unaltered in the mutant compared to parental cells (Loovers *et al.*, 2007). A complication in interpreting data with the Dd5P4 phosphatase mutant is that this phosphatase also possesses a RhoGAP domain, which is highly specific for Rac1 (Loovers *et al.*, 2007). As will be shown further below, constitutively active Rac1 inhibits both phagocytosis and macropinocytosis. The observation that the isolated catalytic domain of the phosphatase fails to rescue the Dd5P4-null mutant raises the possibility that the observed effects of gene disruption on both phagocytosis and macropinocytosis may be mediated by the RhoGAP domain rather than by the 5-phosphatase activity (Loovers *et al.*, 2007).

Taken together, this wealth of results pinpoints the importance of phosphoinositides, in particular changes in PI(4,5)P2 dynamics, for phagocytic uptake, though additional studies are required to better define the role of specific PI classes, the generating enzymes and downstream effectors in regulating the different steps in actin dynamics. The recent generation of a sextuple mutant, in which all PI3K's plus PTEN have been deleted, may help in better defining the role of PLC. Interestingly, the sextuple mutant shows only a mild defect in chemotaxis, suggesting that actin dynamics in response to chemoattractants is essentially unaltered. The mutant grows slowly in axenic medium and on a bacterial lawn, but whether this is due to defective macropinocytosis and phagocytosis has not been reported (Hoeller and Kay, 2007). The emerging picture of phagocytosis control by modulation of PI(4,5)P2 sites in *Dictyostelium* is remarkably similar to what has been described for mammalian phagocytes (Scott *et al.*, 2005).

Phosphoinositides regulate also phagosomal intracellular traffic and phagolysosomal maturation (Rupper *et al.*, 2001b). Transition from acidic to nonacidic postlysosomal vacuoles is delayed upon inhibition of PI3K either with drugs or by gene disruption, suggesting a role for this kinase in intracellular vesicle fusion at later stages of endocytosis. Indeed, both in the double PI3K1/2 mutant and after treatment of control cells with PI3K inhibitors formation of multiparticle spacious phagosomes is delayed and phagosomes remain acidic for longer time than in control cells (Rupper *et al.*, 2001b) (see also Section 2). A similar phenotype has been described for Akt/PKB, a well-known effector of PI3K (Rupper *et al.*, 2001b; Zhou *et al.*, 1995). The mechanism of action of PI3K and Akt in stimulating vesicle maturation is unclear. An artificial increase in phagosomal pH has been shown to rescue formation of spacious phagosomes. It has thus been proposed that PI3K and Akt may favor delivery or removal of ion channels in the maturing phagosome, thus regulating counter ion conductance (Rupper *et al.*, 2001b). A class III PI3K, homologous to yeast Vps34p, named DdPIK5, also exists in *Dictyostelium*, and *DdPIK5* antisense expression inhibits growth on bacterial lawn (Zhou *et al.*, 1995). Whether the growth defect is due to impaired phagocytosis has not been checked.

4.3. Small G proteins of the Ras and Rac families and tyrosine kinases

Dictyostelium cells possess several small G proteins of the Ras, Rac, and Rab families, as well as a large number of both GDP/GTP exchange factors (GEF's) and G-activating proteins (GAP's) (Vlahou and Rivero, 2006; Weeks and Spiegelman, 2003). Rac proteins in particular are well-known regulators of the actin cytoskeleton, by activating WASP/WAVE family proteins and formins (Ridley, 2006), whereas Rab's are master timers of intracellular vesicle traffic and fusion (Zerial and McBride, 2001).

Small G proteins of the Ras family act as molecular switches in signal transduction pathways and have been involved in regulating G protein-dependent and -independent processes, including cell growth and cytokinesis, spontaneous and chemotactic motility, adenylyl cyclase activation, and actin polymerization (Sasaki and Firtel, 2006; Weeks and Spiegelman, 2003).

Of the eight Ras genes present in the *Dictyostelium* genome, only two appear to be involved in phagocytosis, namely, RasS and the Ras-like protein Rap1. RasS-null mutants are characterized by many elongated actin protrusions and are defective in both phagocytosis and macropinocytosis, but display enhanced cell migration (Chubb *et al.*, 2000). The small Ras-like protein Rap1 also regulates phagocytosis, as the phagocytosis rate of both bacteria and latex beads is increased in cells overexpressing a wild-type form of the protein and is decreased upon expression of a dominant-negative form (Seastone *et al.*, 1999). A potential downstrean effector of RasS and Rap1 is the serine/threonine Phg2 kinase, which has been shown to interact *in vitro* with RasS, RasG, and Rap1. Phg2-null mutants display altered actin polymerization and are defective in phagocytosis and in adhesion to hydrophilic, but not hydrophobic, surfaces, similarly to talin- or myo VII-null mutants. They are not defective in macropinocytosis, further supporting a role for this kinase in particle adhesion and phagocytic cup formation (Gebbie *et al.*, 2004).

There are 18 genes encoding Rho GTPases in the *Dictyostelium* genome, all belonging to the Rac subfamily. No homologues of Cdc42 and the founding member Rho have been found (Eichinger *et al.*, 2005; Vlahou and Rivero, 2006). Nine genes, namely, Rac1A to C and RacB, C, E, F1, G, and H, have been studied in detail. RacE, apparently regulates cytokinesis, but not phagocytosis or endocytosis (Larochelle *et al.*, 1997). RacF1 accumulates transiently in phagocytic cup and phagosome, but its ablation has no effect on phagocytosis or endocytosis. Overexpressor, constitutive or dominant-negative mutants were not studied (Rivero *et al.*, 1999). Rac1, RacB, RacC, RacG, and RacH appear to regulate phagocytosis, though with somewhat different effects. The Rac1 members, Rac1A, -B, and -C, display similar phenotypes, both in term of actin dynamics and effects on

phagocytosis (Dumontier *et al.*, 2000). Overexpression of the wild-type forms leads to formation of extended, very mobile filopodia, whereas expression of constitutive active forms stimulates crown-shaped extensions, typical of macropinocytosing cells. Bacterial and yeast phagocytosis are slightly stimulated or inhibited in cells overexpressing wild-type Rac1 or dominant-negative Rac1, respectively. In contrast, strong inhibition was found in cells expressing constitutively active Rac1. Macropinocytosis was also strongly inhibited in the latter cells (Dumontier *et al.*, 2000).

A decrease in phagocytosis rate was found in cells expressing constitutively active as well as dominant-negative RacB (Lee *et al.*, 2003). Extensive actin protrusions were observed in constitutively active RacB cells, but it is open whether the inhibitory effect on phagocytosis is linked to these extensions, since they are not found in dominant-negative mutants.

Overexpression of constitutively active RacC stimulated bacterial phagocytosis, but had the opposite effect on macropinocytosis (Seastone *et al.*, 1998). RacC has been recently shown to regulate actin dynamics through WASP activation and to be also required for PI3K translocation to the membrane (Han *et al.*, 2006).

RacG is targeted to the rim of the progressing phagocytic cup and disappears from the phagosome immediately after its internalization. This GTPase favors formation of actin meshwork via Arp2/3, in a WASP-independent pathway (Somesh *et al.*, 2006b). RacG overexpression or expression of a constitutively active form increased the rate of yeast particle uptake, a process that was counteracted by PI3K inhibitors. RacG ablation has no effect on yeast phagocytosis (Somesh *et al.*, 2006b). Effects on bacterial phagocytosis have not been reported for RacG.

A severe defect in both macropinocytosis and latex beads phagocytosis was observed in cells overexpressing wild-type RacH. RacH-deficient cells display, instead, normal phagocytosis rates, although fluid-phase uptake is reduced by 50%. In contrast to the other *Dictyostelium* Rac GTPases, RacH localizes mostly to ER and Golgi membranes. Thus, the mutant phenotypes are possibly related to altered intracellular vesicular traffic. Indeed formation of large vacuoles and recruitment of vacuolin, a marker of postlysosomal vacuoles, were impaired in the mutant, which also displays reduced acidification (Somesh *et al.*, 2006a). These results establish Rac GTPases as important regulators of the actin cytoskeleton during phagocytosis, both during uptake and during phagosome maturation, but underline also a high degree of redundancy and overlapping functions, which explains the absence of phenotypes when single genes are disrupted.

In contrast to macrophages, where they play a major role, tyrosine kinases have not been involved in *Dictyostelium* phagocytosis, except for a membrane tyrosine kinase-like protein, termed VSK3. VSK3 consists of a single-transmembrane domain, a C terminal catalytic domain and a TIG (immunoglobulin-like fold) domain, typical of the MET kinase family.

The protein localizes to the surface of late endosomes or phagosomes, and its ablation leads to a reduction in bacterial and yeast phagocytosis, not however in macropinocytosis (Fang *et al.*, 2007). Particle uptake is normal in VSK3 knockout mutant, but fusion of internalized phagosomes with endolysosomal vesicles is impaired, suggesting a role for this kinase in phagosomal maturation. The kinase is enzymatically active *in vitro* and the kinase domain is essential for the function of the protein (Fang *et al.*, 2007).

4.4. The Rab family in intracellular phagosome maturation

As mentioned, small G proteins of the Rab family regulate formation of transport vesicles from donor compartments, their traffic on cytoskeleton, and tethering to acceptor compartments (Zerial and McBride, 2001). In the *Dictyostelium* genome, 58 genes encode Rab proteins, most of which fail to undergo expression changes in a DNA microarray comparing cells engaged in phagocytosis versus macropinocytosis, suggesting that the Rab machinery is mostly unaltered in phagocytosis and macropinocytosis (Sillo *et al.*, in press). Only a handful of Rab proteins have been studied at molecular genetic level. Consistent with the previous conclusion, mutations affecting specific Rab's, such as RabD (related to mammalian Rab14) or Rab7, affect both phagocytosis and macropinocytosis (Harris and Cardelli, 2002; Rupper *et al.*, 2001a). Rab7 is recruited to phagosomes immediately after actin uncoating and appears to regulate fusion of early endosomes/phagosomes with a subset of lysosomes-containing α-mannosidase and LmpA, not, however, the vacuolar ATPase (Buczynski *et al.*, 1997a; Laurent *et al.*, 1998; Rupper *et al.*, 2001a). RabD appears, instead, to mediate phagosomal homotypic fusion into multiparticle large phagosomes (Harris and Cardelli, 2002). In contrast to these proteins, the homologue of mammalian Rab11 is located specifically in the contractile vacuole, but expression of a dominant-negative form leads to increased phagocytosis.

A Rab protein specifically acting on phagocytosis, not pinocytosis, is Rab21 (previously termed RabB), belonging to the group V family. Constitutively active or dominant-negative Rab21 increases or decreases, respectively, the uptake rate of yeast particles (Khurana *et al.*, 2005). Rab21 forms a complex with two interactors, a Lim-domain and a Calponin-Lim-domain protein, that activate or inhibit Rab21, respectively. The interactors are slightly enriched in the nascent phagosome, though no enrichment of Rab21 was found (Khurana *et al.*, 2005).

Also in macrophages Rab proteins have been recognized as key factors for phagosome formation and phagolysosome biogenesis although, in contrast to *Dictyostelium*, a specific Rab protein for phagocytosis has not been identified yet. In macrophages, activated Rab5a is recruited rapidly, already during actin assembly, and transiently to newly formed phagosomes. Recruitment of active Rab5a on phagosomes is required for the subsequent

enrollment of Rab7 (Kitano *et al.*, 2008; Roberts *et al.*, 2000; Vieira *et al.*, 2003). Early phagosomes acquire Rab7 from a soluble pool and/or by fusion with Rab7-containing endosomes. Acquisition of Rab7 enables phagosomes in macrophages to fuse with late endocytic compartments, such as late endosomes and lysosomes. Indeed, activated Rab7 recruits on the phagosomal membrane its effector RILP, which is responsible for dynein-dynactin recruitment. Recruitment of motors promotes movement of phagosomes toward the MTOC and formation of tubular extensions, which fuse with late endosomes and lysosomes, allowing acquisition of lysosomal content and maturation of phagosomes into phagolysosomes (Harrison *et al.*, 2004). No RILP homologue has been found in the *Dictyostelium* genome (Bucci, unpublished observations). Phagosomal acquisition of Rab5 in macrophages stimulates phagocytosis of latex beads but not Fcγ or C3 receptor-mediated phagocytosis, indicating a differential regulation of various kinds of phagocytosis (Duclos *et al.*, 2000).

Regulation of Rab function by cytokines has been recently demonstrated. Cytokines IL-6 and IL-12, through the activation of specific kinases regulate the expression of particular endocytic Rab proteins (Bhattacharya *et al.*, 2006). Indeed, IL-6 induces expression of Rab5 through the activation of ERK leading to increased fusion of early endocytic compartments, whereas IL-12 induces Rab7 expression through the activation of p38 MAPK leading to increased lysosomal transport (Bhattacharya *et al.*, 2006). Interestingly, the recruitment of both Rab5 and Rab7 to phagosomes is modulated by the phosphatidylinositol 3-kinase (PI3K) and inhibition of PI3K by wortmannin impairs phagolysosomes biogenesis despite the presence of active Rab7 on phagosomes (Vieira *et al.*, 2003).

Phagosome biogenesis depends on plasma membrane availability and therefore recycling it is thought to be important for phagocytosis. In mammalian cells, endosomal recycling is under the control of Rab4 and Rab11, which function sequentially. Rab4 regulates recycling from sorting endosomes and Rab11 controls transport through the perinuclear endosomal recycling compartment. Active Rab11 has been detected on nascent phagosomes in macrophages, and it is required for rapid recycling and retrieval to the plasma membrane of phagosomal content (Cox *et al.*, 2000; Leiva *et al.*, 2006). Indeed, it has been demonstrated that expression of a dominant-negative Rab11 mutant decreases the rate of transferrin efflux and, as a consequence, impairs FcγR-dependent phagocytosis, while expression of a constitutively active Rab11 mutant causes increased transferrin recycling and enhances phagocytosis (Cox *et al.*, 2000; Leiva *et al.*, 2006).

Also the Rab coupling protein (RCP), which interacts with Rab4 and Rab11, has been detected on the early phagosomal membrane. RCP is localized within particular protein subdomains on the early phagosomal membranes and controls recycling from these membranes. Indeed,

overexpression of RCP stimulates recycling and phagocytosis while expression of a truncated form inhibits them (Damiani *et al.*, 2004).

Proteomic studies have detected also Rab2, Rab5b, Rab5c, Rab9, Rab10, and Rab14 on phagosomes containing latex beads (Garin *et al.*, 2001). The role of Rab2 in promoting engulfment and degradation of apoptotic cells has been investigated only in *Caenorhabditis elegans*, while a detailed study on Rab14 has demonstrated that this Rab protein is important for fusion of the mycobacterial phagosome with early endocytic compartments (Kyei *et al.*, 2006; Mangahas *et al.*, 2008). *Mycobacterium tuberculosis* survives in macrophages arresting phagosomal maturation. Rab14 silencing or expression of Rab14 dominant-negative mutants lead to phagolysosomal maturation of phagosomes containing live mycobacteria, whereas overexpression of Rab14 or of a constitutively active Rab14 mutant blocks maturation of phagosomes containing dead bacteria (Kyei *et al.*, 2006). Rab21 and Rab22 have been initially localized to the early endosomal compartment in mammalian cells and it has been demonstrated that they regulate endocytosis and morphology of sorting early endosomes (Mesa *et al.*, 2001; Simpson *et al.*, 2004). Both proteins in macrophages were transiently recruited on latex beads containing phagosomes similarly to Rab5 (Roberts *et al.*, 2006). Interestingly, on *M. tuberculosis*-containing phagosomes Rab22 was retained and enriched and, similarly to Rab14, maturation to phagolysosomes was impaired. Silencing of Rab22 increased phagosome maturation whereas overexpression of a constitutively active mutant prevented maturation of phagosomes-containing dead bacteria (Roberts *et al.*, 2006). Therefore, the presence of active Rab14 and active Rab22 on phagosomes in macrophages seems to be important to inhibit or delay phagolysosomal biogenesis.

Recently, comparison of phagosomes containing wild-type and a non-invasive mutant of *Salmonella enterica* serovar *typhimurium* in macrophages has allowed identification of 18 Rab proteins (included the ones previously shown as phagosome-associated), whose kinetics of association were recorded (Smith *et al.*, 2007). Among the newly identified there are Rab8b, Rab13, Rab23, and Rab35 (Smith *et al.*, 2007). Beside the numerous differences in the amount and in the kinetics of association/dissociation of certain Rab proteins in phagosomes containing the wild type or the mutant *Salmonella*, this study demonstrates that formation and maturation of phagosomes into phagolysosomes is a very complex event controlled by a network of Rab GTPases. Moreover, this study identified Rab23 and Rab35 as additional Rab proteins whose function is necessary for phagosome maturation (Smith *et al.*, 2007). It is therefore now clear that biogenesis of phagosomes and phagolysosomes in macrophages is a process that involves several different Rab GTPases and their effectors and that cannot be explained only by the single transition between Rab5 and Rab7. We are, therefore, still far away from its complete comprehension although several

players have now been identified. Because of the large number of genes encoding Rab proteins in the *Dictyostelium* genome, these cells would be excellent candidates for a systematic molecular genetic study of the Rab family in phagocytosis.

5. Host–Pathogen Interactions: A Versatile New Model Host

Phagocytosis is exploited by invasive bacteria for entering the cell and proliferating in protected intracellular niches. Having examined the molecular mechanisms underlying phagocytosis, in this section we will review briefly studies on *Dictyostelium* cell–pathogen interactions that have shed light on genetic host determinants of susceptibility or resistance to infection by invasive bacteria. Although it was known from the early report of Depraitère and Darmon (1978) that a few bacteria were pathogenic for *Dictyostelium*, the system has emerged as a suitable experimental model for bacterial infections only in recent years, the first two pioneering reports, both with *Legionella pneumophila*, being only 8 years old (Hagele *et al.*, 2000; Solomon *et al.*, 2000).

Shortly later, research was extended to *Mycobacterium avium* and *Mycobacterium marinum* (Skriwan *et al.*, 2002; Solomon *et al.*, 2003), *P. aeruginosa* (Cosson *et al.*, 2002; Pukatzki *et al.*, 2002), *Vibrio cholerae* (Pukatzki *et al.*, 2006), *K. pneumoniae* (Benghezal *et al.*, 2006), and to nonculturable endosymbionts *(Neochlamydia sp.TUME1* and *Parachlamydia sp.UWE25)* of *Acanthamoeba* (Skriwan *et al.*, 2002). Worth mentioning is also *Criptococcus neoformans*, an environmental fungus that can cause meningitis and is also phagocytosed by *Dictyostelium* (Steenbergen *et al.*, 2003). Very recently, evidence has been provided that *Neisseria meningitidis*, also a potent agent of fulminating meningitis, is phagocytosed by *Dictyostelium* cells and is, at least in part, pathogenic (Colucci *et al.*, 2008).

5.1. Resistance/susceptibility genes of the host to infection by microbes

As a soil amoeba, *Dictyostelium* can be natural host of opportunistic bacteria, and may thus have developed defence mechanisms against aggressive microbes. Unlike other amoebae, such as *Acanthamoeba castellanii* or *Hartmanella vermiformis*, *Dictyostelium* is amenable to genetic analysis. The availability of several knockout mutants and the possibility of easily screening for novel mutants with variable resistance to infection make these cells attractive for identifying host defence factors. Table 6.1 is a summary of

Table 6.1 Host cell factors that affect *Dictyostelium*–pathogen interactions

Host cell factor	Exp. Approach	Effects on infection	Pathogen	References
F-actin	Inhibitors	Uptake down/ growth up	L.p.	Balest and Bozzaro (unpublished results) and Lu and Clarke (2005)
α-Actinin/ ABP120	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
Coronin	Knockout	Uptake down/ growth normal	L.p., M.m.	Fajardo *et al.* (2004) and Solomon *et al.* (2003)
Comitin	Knockout	Uptake down/ growth up	L.p.	Schreiner *et al.* (2003)
Myosin1(A/B)	Knockout	Uptake normal/ growth up	L.p.	Solomon *et al.* (2000)
Profilin I/II	Knockout	Uptake normal/ growth up	L.p.	Balest and Bozzaro (unpublished results) and Hägele *et al.* (2000)
Daip1	Knockout	Uptake down/ growth normal	L.p.	Fajardo *et al.* (2004)
Villidin	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
Lim C/D	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
Gβ subunit	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
RacH	Knockout	Uptake down/ growth up	L.p., M.m.	Balest and Bozzaro (unpublished results) and Hagedorn and Soldati (2007)
PLC	Inhibitors	Uptake down/n.t.	L.p.	Fajardo *et al.* (2004)
Calcium level	Inhibitors	Uptake down/n.t.	L.p.	Fajardo *et al.* (2004)

Calnexin	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
Calreticulin	Knockout	Uptake down/ growth down	L.p.	Fajardo *et al.* (2004)
PI3K	Inhibitors	Uptake down/ growth up	L.p.	Balest and Bozzaro (unpublished results) and Weber *et al.* (2006)
PI3K1/2	Knockout	Uptake down/ growth up	L.p.	Balest and Bozzaro (unpublished results) and Weber *et al.* (2006)
Phg1	Knockout	Uptake normal/ growth up	K.p.	Benghezal *et al.* (2006)
Nramp1	Knockout	Uptake normal/ growth up	L.p., M.a., M.m.	Peracino *et al.* (2006) and Soldati (personal communication)
	Overexpression	Uptake normal/ growth down	L.p., M.a.	Peracino *et al.* (2006)
Vacuolin B	Knockout	Uptake normal/ growth down	M.m.	Hagedorn and Soldati (2007)
RtoA	Knockout	Uptake normal/ growth down	L.p.	Li *et al.* (2005)
Kil1	Knockout	Uptake normal/ growth up	K.p.	Benghezal *et al.* (2006)
	Overexpression	Uptake normal/ growth down	K.p.	Benghezal *et al.* (2006)
TirA	Knockout	N.t./growth up	L.p.	Chen *et al.* (2007)
AMPK	Overexpression/ antisense	Growth up	L.p.	Francione and Fisher (personal communication)

Pathogen uptake and intracellular growth in *Dictyostelium* mutants or upon treatment of wild-type cells with inhibitors (F-actin: cytochalasin A, latrunculin; PLC: U73122; intracellular calcium levels: BAPTA-AM, Thapsigargin; PI3K: wortmannin, LY24002).
N.t., not tested; L.p., *L. pneumophila*; K.p., *K. pneumoniae*; M.a, *M. avium*; M.m., *M. marinum*.

Dictyostelium genes, which have been found to affect resistance to infection by different pathogens.

The system can also be used for mapping microbial virulence genes, as shown for *Pseudomonas*, virulent *Vibrio* strains (Alibaud *et al.*, 2008; Cosson *et al.*, 2002; Pukatzki *et al.*, 2002, 2006), *Legionella* (Hagele *et al.*, 2000; Weber *et al.*, 2006), *Klebsiella* (Benghezal *et al.*, 2006), and *Neisseria* (Colucci *et al.*, 2008). The state of the art has been reviewed recently (Steinert and Heuner, 2005), therefore we will mainly examine some open questions and the latest developments on host defence factors identified in infection studies with *Legionella, Mycobacteria*, and *Klebsiella*. Microbial virulence genes will not be discussed.

In contrast to *Legionella* and *Mycobacteria*, *K. pneumoniae* is not pathogenic for wild-type *Dictyostelium* cells. Sensitive to *K. pneumoniae* is, instead, the Phg1-null mutant (Benghezal *et al.*, 2006). As mentioned in Section 3.1, the Phg-1 null mutant is defective in latex beads, but not bacteria uptake. *K. pneumoniae* is no exception, but the bacteria survive intracellularly. Suppression genetics has led to the identification of a second gene, *kil1*, whose overexpression in the Phg1-background rescues the mutant. The *kil1* protein product is a sulfotransferase involved in sulfation of sugars and glycoproteins; sulfated glycoproteins are undetectable in the mutant. It has been suggested that Phg1 and Kil1 might be both involved in the delivery of sulfated lysosomal enzymes to the phagosome (Benghezal *et al.*, 2006). These should, however, be rather specific for *Klebsiella*, as other bacterial species are killed by the double mutant.

The dynamics of infection has been most studied with *Legionella* pneumophyla and Mycobacteria (*M. avium* or *marinum*). In contrast to Mycobacteria, which are phagocytosed by *Dictyostelium* cells also under shaking, *Legionella* is taken up, and to a rather limited extent, only if coincubated with cells in dishes; cocentrifugation is often used to improve the uptake (Solomon *et al.*, 2000). There is some evidence that *Legionella* uptake occurs by macropinocytosis, rather than phagocytosis (Balest and Bozzaro, unpublished results).

Both *Legionella* and Mycobacteria survive and proliferate intracellularly, following a route somewhat similar to what has been described for macrophages. In the case of Mycobacteria, early recruitment of V-H^+ ATPase to phagosome and acidification are strongly reduced, suggesting that phagosomal maturation is arrested or bypassed (Hagedorn and Soldati, 2007). Reduced acidification of the *Mycobacterium*-containing vacuole has also been reported for macrophages (Sturgill-Koszycki *et al.*, 1994). At later stages of infection, the Mycobacteria proliferate in neutral postlysosomal, vacuolin-positive spacious vacuoles, which however do not fuse with the plasma membrane, but undergo rupture to deliver the bacteria in the cytosol (Hagedorn and Soldati, 2007). How the bacteria escape from the cytosol is unclear.

Intracellular growth, but not uptake, is enhanced in Nramp1-null mutant for both *M. avium* (Peracino *et al.*, 2006) and *M. marinum* (Soldati, personal communication), suggesting that control of iron homeostasis is important for establishing a friendly environment for Mycobacteria (see also next section). Remarkably, intracellular growth is also enhanced in a RacH-null mutant, but is inhibited in a vacuolin B-null mutant, in which acidification and normal phagosomal maturation are somewhat restored (Hagedorn and Soldati, 2007). Enhanced intracellular growth in the RacH-null mutant may be due to the defective acidification that has been reported for this mutant (Somesh *et al.*, 2006a, and Section 4.3). The vacuolin B-null mutant is characterized by very large vacuoles and impairment in excretion of postlysosomal debris (see Section 3.3). Because of structural similarities with caveolin, it has been suggested that lack of the vacuolin coat may facilitate fusion/fission of the Mycobacteria-containing phagosome with vesicles of the endocytic pathway, thus favoring recruitment of vacuolar ATPase and lysosomal enzymes, which would be counteractive for mycobacterial survival (Hagedorn and Soldati, 2007).

Legionella infection in the *Dictyostelium* host system has been extensively studied. The *L. pneumophila* genome analysis has identified several homologues of eukaryotic genes, and it has been speculated that the respective proteins may allow *Legionella* to communicate with eukaryotic cells (Jules and Buchrieser, 2007). Moreover, the analysis of the *Dictyostelium* transcriptome upon infection with *L. pneumophila* has provided a better understanding of the manifold host cell responses (Farbrother *et al.*, 2006). Functional annotation of the differential regulated genes revealed that by establishing its replicative niche *Legionella* not only interferes with bacterial degradation and intracellular vesicle fusion and destination but also profoundly influences and exploits the metabolism of its host. Functional studies revealed that uptake requires polymerization of the actin cytoskeleton, and is reduced in some mutants defective in actin-binding proteins or myosin I (Table 6.1 and Fajardo *et al.*, 2004; Lu and Clarke, 2005; Steinert and Heuner, 2005). Following a short phase of rapid movement, apparently along microtubules, the *Legionella*-containing vesicle is rapidly decorated with calnexin, with calreticulin, and with the protein marker HDEL, suggesting close association with the ER and the ER/preGolgi intermediate compartment (Fajardo *et al.*, 2004; Li *et al.*, 2005; Lu and Clarke, 2005). During the following 2–3 h postinfection, the *Legionella*-containing vesicle transforms into a large replicative vacuole still decorated with calnexin and calreticulin (Fajardo *et al.*, 2004; Lu and Clarke, 2005).

These data are in agreement with studies in macrophages and support the notion that maturation of the *Legionella* replicative vacuole occurs by fusion with secretory vesicles recruited from the ER (Kagan and Roy, 2002; Swanson and Isberg, 1995). Similarly to macrophages, also in Dictyostelium the Legionella-containing vacuole recruits mitochondria within 30 min post-infection (Francione and Fisher, personal communication). In contrast

to nonpathogenic bacteria, whose phagosomes rapidly fuse with V-H^+ ATPase, delivery of the vacuolar ATPase to the *Legionella* vacuole has not been observed during the first hours of infection (Balest and Bozzaro, unpublished results; Lu and Clarke, 2005). A partial recruitment has been reported if TAMRA- or TRITC-labeled *Legionella* are used for infection (Peracino *et al.*, 2006). However, *Legionella* viability is strongly reduced upon fluorescence labeling, thus the V-H^+-ATPase-decorated vesicles most likely contain sick or dead bacteria. In addition to the vacuolar ATPase, no fusion with lysosomal vesicles, assessed by using antibodies against lysosomal markers (DdLIMP), has been observed, supporting the notion that *Legionella* avoids quite rapidly the endosomal maturation pathway (Lu and Clarke, 2005). Similarly to macrophages (Sturgill-Koszycki and Swanson, 2000), however, the vacuole transforms late during infection into a ''spacious'' replicative vacuole decorated with the V-H^+ ATPase, and thus presumably acidic (Balest and Bozzaro, unpublished results).

Phosphoinositide metabolism appears to play a major role for the establishment of the replicative vacuole. Intracellular growth of *Legionella* is strongly enhanced by PI3K inhibitors or in the PI3K1/2-null mutant (Weber *et al.*, 2006). Initial docking and fusion of the *Legionella*-containing vacuole (LCV) with the ER are unaffected, but formation of spacious vacuoles is strongly reduced in cells lacking functional PI3K (Weber *et al.*, 2006). The described effect is reminiscent of the inhibition of spacious postlysosomal vacuoles by PI3K inhibitors during phagosomal maturation (see Section 4.3), and suggests that any agent that delays or blocks the endocytic pathway favors the establishment of a replicative niche. Weber *et al.* (2006), however, provide some evidence that *Legionella* subverts the host cell PI metabolism, favoring enrichment of PI(4)P in the LCV. This phospholipid anchors specifically SidC, one of the secreted protein substrates of the type IV Intracellular multiplication/Defective organelle trafficking (Icm/Dot) type IV secretion system. The *Legionella* Icm/Dot type IV secretion system is a conjugation apparatus that is required for vesicle traffic and formation of the LCV, and is essential for *Legionella* pathogenicity, though not required for intracellular bacterial replication (Hilbi *et al.*, 2001; Vogel *et al.*, 1998). Icm/Dot substrates are of particular interest. SidM and LidA target the mammalian Rab1, a small GTPase regulating ER-to-Golgi traffic. RalF recruits and activates ADP-ribosylation factor 1 (Arf1), a small GTPase involved in retrograde vesicle transport from Golgi apparatus to ER (Derré and Isberg, 2005; Nagai *et al.*, 2002). The function of the SidC protein is unknown. In *Legionella*-infected *Dictyostelium* cells as well as macrophages, the protein localizes to the cytoplasmic surface of vacuoles. In the absence of functional PI3K, SidC recruitment to LCV is increased, suggesting that PI(4)P sites are enriched. Interestingly, PI(4)P sites are also enriched in vesicles harbouring wild-type *Legionella* compared to Δ-*icmT*/*Dot Legionella* mutant, suggesting that the generation of PI(4)P sites is, at least in part, Icm/Dot dependent (Weber *et al.*, 2006).

Dictyostelium cells possess cytosolic Toll Interleukin1 Receptor (TIR)-domain proteins (Chen *et al.*, 2007). The *tirA* gene, encoding one such protein, is highly expressed in growth phase cells and in "sentinel cells," the small cell subpopulation in tight aggregates and slugs, which has conserved the ability to phagocytose and is thought to act as specialized neutrophil-like clearer of pathogens in the multicellular organism (see Section 1). *TirA* gene disruption results in cells forming minute colonies on nonpathogenic bacterial lawn and displaying enhanced killing by *Legionella* (Chen *et al.*, 2007). Remarkably, the viability of the mutant cells is reduced upon growth on nonpathogenic bacteria, suggesting a potential role of TirA in the cell response to grow on bacteria. Consistent with mitochondria recruitment to the replicative vacuole, mitochondrially-diseased cells are more susceptible to infection. Increased susceptibility appears to be linked to chronic AMPK activation, and can be suppressed by antisense inhibiting endogenous AMPK (Francione and Fisher, personal communication). Similarly to Mycobacteria, intracellular growth of *Legionella* is enhanced in Nramp1-null mutants (Peracino *et al.*, 2006), establishing this protein as a crucial *Dictyostelium* resistance factor to infection against invasive bacteria.

5.2. The Nramp family in *Dictyostelium* and Nramp1 as host defence factor

The Nramp1 has been widely studied in macrophages, following his discovery as a genetic factor responsible for macrophage innate resistance to various intracellular pathogens (Vidal *et al.*, 1993). It is part of a larger Nramp (or solute carrier 11, SLC11) family, whose members confer resistance to metal chelation in yeast, mediate dietary iron uptake in the apical membrane of epithelial cells of the brush border, and iron supply to erythrocytes (for reviews, see Forbes and Gros, 2001; Nevo and Nelson, 2006). Distant orthologs of Nramp exist in Gram-positive and Gram-negative bacteria, the prototype probably being proton-dependent manganese transporters (Richer *et al.*, 2003). Based on sequence conservation of the overall membrane structure and functional studies in different model systems, it is established that Nramp proteins are H^+-dependent divalent metal transporters (for a recent review, see Courville *et al.*, 2006).

Two Nramp parologs, Nramp1 and Nramp2, exist in mammals. Nramp1 is selectively expressed in the endosomal pathway of professional phagocytes, whereas Nramp2 is expressed on the cell surface or subcellular membrane compartments of most tissues. Mutations in Nramp2 functions have been discovered in patients suffering iron disorders, such as microcytic anemia or serum and hepatic overload. Nramp2 is essential for intenstinal iron transport in rodents (Gunshin *et al.*, 2005; Su *et al.*, 1998). Mutations in Nramp1 have, instead, been linked to innate susceptibility to mycobacterial diseases, *Salmonella* infection and to autoimmune diseases (Bellamy, 2003; Blackwell *et al.*, 2003; Jabado *et al.*, 2003; Malik *et al.*, 2005). *Dictyostelium*

cells possess two genes, Nramp1 and Nramp2, Nramp1 being closer to mammalian Nramp1 (Peracino *et al.*, 2006). The Nramp2 gene is intronless, whereas the Nramp1 gene contains a single intron at the N-terminus, at a site conserved in all higher eukaryotes (Richer *et al.*, 2003).

Nramp1 is expressed exclusively on the membrane of endolysosomal vesicles and in the Golgi, and starts to be recruited to macropinosomes and phagosomes, containing nonpathogenic bacteria, within 2 min from uptake (Peracino *et al.*, 2006). Nramp2 decorates, instead, exclusively the tubular network of the contractile vacuole (Peracino and Bozzaro, unpublished results). Nramp1-null mutants, as mentioned in the previous section, are more susceptible to infection by both *Legionella* and Mycobacteria than the parental wild-type strain. Phagocytosis of pathogenic as well as nonpathogenic bacteria is only slightly affected in the mutant, but intracellular growth of *Legionella* and Mycobacteria is enhanced. Constitutive Nramp1 expression rescues the mutants and protects effectively against *Legionella* infection (Peracino *et al.*, 2006). Nramp2-null mutants have been recently generated, but they have not been tested so far for infection.

Iron transport studies with isolated phagosomes have provided evidence in *Dictyostelium* for H^+-dependent and -independent iron transport via Nramp1, suggesting that the protective role of Nramp1 is due to iron depletion from the *Legionella*- or *Mycobacterium*-containing vacuole (Peracino *et al.*, 2006). Iron is an essential metal for all cells and is known that *Legionella* and Mycobacteria accumulate a large amount of iron. For Nramp1 to deplete the phagosomal lumen of iron, a functional V-H^+ ATPase is essential. The vacuolar ATPase fails, however, to be recruited to the LCV during the first hours of infection (Lu and Clarke, 2005; Peracino and Bozzaro, unpublished). Whether Nramp1 is normally recruited to the LCV is unknown. A constitutively expressed GFP fusion protein decorates about 40% of LCV's from 2 to 24 hours of infection (Peracino and Bozzaro, unpublished). We have found, however, that *Legionella* represses expression of the endogenous Nramp1 gene, such that 12-h postinfection no RNA is detected (Peracino *et al.*, 2006). It is thus possible that when the vacuolar ATPase is recruited to the *Legionella*-reproductive vacuole, the endogenous Nramp1 protein is no more expressed, and iron cannot be transported out of the vacuole. Expression of Nramp1 from a constitutive promoter circumvents the *Legionella*-induced repression of the endogenous promoter, favoring iron transport, and thus inhibiting *Legionella* growth.

6. Concluding Remarks

Genetics is clearly the hallmark of *Dictyostelium* as model for phagocytosis and host–pathogen interactions, when compared to macrophages. The ease with which one can generate mutants and analyze them with all sorts of

cell biological tools is one of the advantages offered by this amoeba, and explains the explosion of activity in the field of phagocytosis and host–pathogen interactions in the last 10 years. Recent proteomic and microarray studies have highlighted several new proteins/genes and biological processes somewhat linked with phagocytosis, which can be investigated in the near future. In addition, in-depth dissection of the well-established regulatory pathways discussed above will be made possible by novel mutants generated recently in different labs.

Further studies with cytoskeletal proteins may help elucidating one of the black boxes in phagocytosis, namely, which factors are involved in closure of the phagocytic cup. Most Rab proteins still await genetic analysis in *Dictyostelium*, and the understanding of intracellular traffic control by Rabs also in mammalian cells would tremendously profit from this analysis. The repertoire of *Dictyostelium* defence mechanisms is largely unknown and its investigation may lead to identification of novel antibacterial peptides. The only drawback for infection studies is that cells do not survive at temperatures above 27 °C, and this may be critical for pathogens encoding virulence factors expressed only at 37 °C. In spite of this, it is possible to imagine that *Dictyostelium* cells may be used as easy and cheap experimental model for early screening of novel drugs against invasive bacteria.

ACKNOWLEDGMENTS

Work in the laboratory of SB was supported by funds of the Italian Ministry of University and Research (PRIN program) and the Piedmont Region (RSF and RSA projects).

REFERENCES

Aizawa, H., Sutoh, K., and Yahara, I. (1996). Overexpression of cofilin stimulates bundling of actin filaments, membrane ruffling, and cell movement in Dictyostelium. *J. Cell Biol.* **132,** 335–344.

Aizawa, H., Fukui, Y., and Yahara, I. (1997). Live dynamics of Dictyostelium cofilin suggests a role in remodeling actin latticework into bundles. *J. Cell Sci.* **110,** 2333–2344.

Alibaud, L., Koehler, T., Coudray, A., Prigent-Combaret, C., Bergeret, E., Perrin, J., and Benghezal, M. (2008). Pseudomonas aeruginosa virulence genes identified in a Dictyostelium host model. *Cell Microbiol.* **10,** 729–740.

Aubry, L., Klein, G., Martiel, J. L., and Satre, M. (1993). Kinetics of endosomal pH evolution in Dictyostelium discoideum amoebae – study by fluorescence spectroscopy. *J. Cell Sci.* **105,** 861–866.

Bellamy, R. (2003). Susceptibility to mycobacterial infections: The importance of host genetics. *Genes Immun.* **4,** 4–11.

Benghezal, M., Cornillon, S., Gebbie, L., Alibaud, L., Bruckert, F., Letourneur, F., *et al.* (2003). Synergistic control of cellular adhesion by transmembrane 9 proteins. *Mol. Biol. Cell* **14,** 2890–2899.

Benghezal, M., Fauvarque, M. O., Tournebize, R., Froquet, R., Marchetti, A., Bergeret, E., Lardy, B., Klein, G., Sansonetti, P., Charette, J. S., and Cosson, P. (2006). Specific host genes required for the killing of Klebsiella bacteria by phagocytes. *Cell. Microbiol.* **8,** 139–148.

Bhattacharya, M., Ojha, N., Solanki, S., Mukhopadhyay, C. K., Madan, R., Patel, N., Krishnamurthy, G., Kumar, S., Basu, S. K., and Mukhopadhyay, A. (2006). IL-6 and IL-12 specifically regulate the expression of Rab5 and Rab7 via distinct signaling pathways. *EMBO J.* **25,** 2878–2888.

Blackwell, J. M., Searle, S., Mohamed, H., and White, J. K. (2003). Divalent cation transport and susceptibility to infectious and autoimmune disease: Continuation of the Ity/Lsh/Bcg/Nramp1/Slc11a1 gene story. *Immunol. Lett.* **85,** 197–203.

Blanc, C., Charette, S., Cherix, N., Lefkir, Y., Cosson, P., and Letourneur, F. (2005). A novel phosphatidylinositol 4,5-bisphosphate-binding domain targeting the Phg2 kinase to the membrane in Dictyostelium cells. *Eur. J. Cell Biol.* **84,** 951–960.

Bogdanovic, A., Bennett, N., Kieffer, S., Louwagie, M., Morio, T., Garin, J., Satr, M., and Bruckert, F. (2002). Syntaxin 7, syntaxin 8, Vti1 and VAMP7 (Vesicle-associated membrane protein 7) form an active SNARE complex for early macropinocytic compartment fusion in Dictyostelium discoideum. *Biochem. J.* **368,** 29–39.

Bozzaro, S., and Gerisch, G. (1978). Contact sites in aggregating cells of Polysphondylium pallidum. *J. Mol. Biol.* **120,** 265–279.

Bozzaro, S., and Roseman, S. (1983a). Adhesion of Dictyostelium discoideum cells to carbohydrates immobilized in polyacrylamide gels. II. Effect of D-glucoside derivatives on development. *J. Biol. Chem.* **258,** 13890–13897.

Bozzaro, S., and Roseman, S. (1983b). Adhesion of Dictyostelium discoideum cells to carbohydrates immobilized in polyacrylamide gels. I. Evidence for three sugar-specific cell surface recptors. *J. Biol. Chem.* **258,** 13882–13889.

Browning, D. D., and O'Day, D. H. (1995). G protein function during biomembrane fusion in Dictyostelium: Presence and importance of a Galphas subunit during fertilization and phagocytosis. *Exp. Cell Res.* **219,** 709–716.

Buczynski, G., Bush, J., Zhang, L. Y., Rodriguez-Paris, J., and Cardelli, J. (1997a). Evidence for a recycling role for Rab7 in regulating a late step in endocytosis and in retention of lysosomal enzymes in Dictyostelium discoideum. *Mol. Biol. Cell* **8,** 1343–1360.

Buczynski, G., Grove, B., Nomura, A., Kleve, M., Bush, J., Firtel, R. A., and Cardelli, J. (1997b). Inactivation of two Dictyostelium discoideum genes, DdPIK1 and DdPIK2, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. *J. Cell Biol.* **136,** 1271–1286.

Ceccarelli, A., and Bozzaro, S. (1992). Selection of mutants defective in binding to immobilized carbohydrates in Dictyostelium discoideum. *Anim. Biol.* **1,** 59–68.

Celli, J., and Finlay, B. B. (2002). Bacterial avoidance of phagocytosis. *Trends Microbiol.* **10,** 232–237.

Chang, Y., and Finnemann, S. C. (2007). Tetraspanin CD81 is required for the alpha v beta5-integrin-dependent particle-binding step of RPE phagocytosis. *J. Cell Sci.* **120,** 3053–3063.

Charette, S., and Cosson, P. (2007). A LYST/beige homolog is involved in biogenesis of Dictyostelium secretory lysozymes. *J. Cell Sci.* **120,** 2338–2343.

Chen, G., Zhuchenko, O., and Kuspa, A. (2007). Immune-like phagocyte activity in the social amoeba. *Science* **317,** 681.

Chia, C. P., and Luna, E. (1989). Phagocytosis in Dictyostelium discoideum is inhibited by antibodies directed primarily against common carbohydrate epitopes pf a major cell surface membrane glycoprotein. *Exp. Cell. Res* **181,** 11–26.

Chia, C. P., Gomathinayagam, S., Schmaltz, R. J., and Smoyer, L. K. (2005). Glycoprotein gp130 of Dictyostelium discoideum influences macropinocytosis and adhesion. *Mol. Biol. Cell* **16,** 2681–2693.

Chubb, J. R., Wilkins, A., Thomas, G. M., and Insall, R. H. (2000). The Dictyostelium RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *J. Cell Sci.* **113,** 709–719.

Clarke, M., and Maddera, L. (2006). Phagocyte meets prey – Uptake, internalization, and killing of bacteria by Dictyostelium amoebae. *Eur. J. Cell Biol.* **85,** 1001–1010.

Clarke, M., Kohler, J., Arana, Q., Liu, T. Y., Heuser, J., and Gerisch, G. (2002a). Dynamics of the vacuolar H+ -ATPase in the contractile vacuole complex and the endosomal pathway of Dictyostelium cells. *J. Cell Sci.* **115,** 2893–2905.

Clarke, M., Kohler, J., Heuser, J., and Gerisch, G. (2002b). Endosome fusion and microtubule-based dynamics in the early endocytic pathway of Dictyostelium. *Traffic* **3,** 791–800.

Cohen, C. J., Bacon, R., Clarke, M., Joiner, K., and Mellman, I. (1994). Dictyostelium discoideum mutants with conditional defects in phagocytosis. *J. Cell Biol.* **126,** 955–966.

Colucci, A. M. R., Peracino, B., Bozzaro, S., Alifano, F., and Bucci, C. (2008). Dictyostelium discoideum as a model host for meningococcal pathogenesis. *Med. Sci. Mon.* **14,** BR134–BR140.

Cornillon, S., Pech, E., Benghezal, M., Ravanel, K., Gaynor, E., Letourneur, F., Bruckert, F., and Cosson, P. (2000). Phg1p is a nine-transmembrane protein superfamily member involved in Dictyostelium adhesion and phagocytosis. *J. Biol. Chem.* **275,** 34287–34292.

Cornillon, S., Dubois, A., Bruckert, F., Lefkir, Y., Marchetti, A., Benghezal, M., DeLozanne, A., Letourneur, F., and Cosson, P. (2002). Two members of the beige/CHS (BEACH) family are involved at different steps of the endocytic pathway in Dictyostelium. *J. Cell. Sci.* **115,** 737–744.

Cornillon, S., Gebbie, L., Benghezal, M., Nair, P., Keller, S., Wehrle-Haller, B., Charette, S. J., Bruckert, F., Letourneur, F., and Cosson, P. (2006). An adhesion molecule in free-living Dictyostelium amoebae with integrin beta features. *EMBO Rep.* **7,** 617–621.

Cosson, P., Zulianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., Van Delden, C., Curty, L. K., and Koehler, T. (2002). Pseudomonas aeruginosa virulence analyzed in a Dictyostelium discoideum host system. *J. Bacteriol.* **184,** 3027–3033.

Courville, P., Chaloupka, R., and Cellier, M. F. (2006). Recent progress in structure-function analyses of Nramp proton-dependent metal-ion transporters. *Biochem. Cell Biol.* **84,** 960–978.

Cox, D., Lee, D. J., Dale, B. M., Calafat, J., and Greenberg, S. (2000). A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis. *Proc. Natl. Acad. Sci. USA* **97,** 680–685.

Damiani, M. T., Pavarotti, M., Leiva, N., Lindsay, A. J., McCaffrey, M. W., and Colombo, M. I. (2004). Rab coupling protein associates with phagosomes and regulates recycling from the phagosomal compartment. *Traffic* **5,** 785–797.

de Hostos, E. L., Bradtke, B., Lottspeich, F., Guggenheim, R., and Gerisch, G. (1991). Coronin, an actin binding protein of Dictyostelium discoideum localized to cell surface projections, has sequence similarities to G protein beta subunits. *EMBO J.* **10,** 4097–4104.

de la Roche, M., Mahasneh, A., Lee, S. F., Rivero, F., and Cote, G. P. (2005). Cellular distribution and functions of wild-type and constitutively activated Dictyostelium PakB. *Mol. Biol. Cell* **16,** 238–247.

Depraitere, C., and Darmon, M. (1978). Croissance de l'amibe sociale Dictyostelium discoideum sur differentes especes bacteriennes. *Ann. Microbiol. (Institute Pasteur)* **129B,** 451–461.

Derré, I., and Isberg, R. (2005). LidA, a translocated substrate of the Legionella pneumophila type IV secretion system, interferes with the early secretory pathway. *Infect. Immun.* **73,** 4370–4380.

Diez, S., Gerisch, G., Anderson, K., Muller-Taubenberger, A., and Bretschneider, T. (2005). Subsecond reorganization of the actin network in cell motility and chemotaxis. *Proc. Natl. Acad. Sci. USA* **102,** 7601–7606.

Dormann, D., Weijer, G., Dowler, S., and Weijer, C. J. (2004). *In vivo* analysis of 3-phosphoinositide dynamics during Dictyostelium in phagocytosis and chemotaxis. *J. Cell Sci.* **117,** 6497–6509.

Downes, C. P., Gray, A., and Lucocq, J. M. (2005). Probing phosphoinositide functions in signaling and membrane traffic. *Trends Cell Biol.* **15,** 259–268.

Drayer, A. L., and van Haastert, P. J. M. (1992). Molecular cloning and expression of a phosphoinositide-specific phospholipase C of Dictyostelium discoideum. *J. Biol. Chem.* **267,** 18387–18392.

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H., and Desjardins, M. (2000). Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages. *J. Cell Sci.* **113,** 3531–3541.

Duerrwang, U., Fujita-Becker, S., Erent, M., Kull, F. J., Tsiavaliaris, G., Geeves, M. A., and Manstein, D. J. (2006). Dictyostelium myosin-IE is a fast molecular motor involved in phagocytosis. *J. Cell Sci.* **119,** 550–558.

Duhon, D., and Cardelli, J. (2002). The regulation of phagosome maturation in Dictyostelium. *J. Muscle Res. Cell Motil.* **23,** 803–808.

Dumontier, M., Hocht, P., Mintert, U., and Faix, J. (2000). Rac1 GTPases control filopodia formation, cell motility, endocytosis, cytokinesis and development in Dictyostelium. *J. Cell Sci.* **113,** 2253–2265.

Eichinger, L., and Rivero, F. (2006). ''Methods in Molecular Biology - Dictyostelium discoideum Protocols.'' 346, Humana Press, Totowa, NJ.

Eichinger, L., Pachebat, J. A., Glockner, G., Rajandream, M. A., Sucgang, R., and Berriman, M. (2005). The genome of the social amoeba Dictyostelium discoideum. *Nature* **435,** 43–57.

Faix, J., and Grosse, R. (2006). Staying in shape with formins. *Dev. Cell* **10,** 693–706.

Fajardo, M., Schleicher, M., Noegel, A., Bozzaro, S., Killinger, S., Heuner, K., Hacker, J., and Steinert, M. (2004). Calnexin, calreticulin and cytoskeleton-associated proteins modulate uptake and growth of Legionella pneumophila in Dictyostelium discoideum. *Microbiology* **150,** 2825–2835.

Fang, J., Brzostowski, J. A., Ou, S., Isik, N., Nair, V., and Jin, T. (2007). A vesicle surface tyrosine kinase regulates phagosome maturation. *J. Cell Biol.* **178,** 411–423.

Farbrother, P., Wagner, C., Na, J. B., Tunggal, B., Morio, T., Urushihara, H., Tanaka, Y., Schleicher, M., Steiner, M., and Eichinger, L. (2006). Dictyostelium transcriptional host cell response upon infection with Legionella. *Cell. Microbiol.* **8,** 438–456.

Favard-Sereno, C., Ludosky, M. A., and Ryter, A. (1981). Freeze-fracture study of phagocytosis in Dictyostelium discoideum. *J. Cell Sci.* **51,** 63–84.

Fey, P., Stephens, S., Titus, M. A., and Chisholm, R. L. (2002). SadA, a novel adhesion receptor in Dictyostelium. *J. Cell Biol.* **159,** 1109–1119.

Fischbach, A., Adelt, S., Muller, A., and Vogel, G. (2006). Disruption of inositol biosynthesis through targeted mutagenesis in Dictyostelium discoideum: Generation and characterization of inositol-auxotrophic mutants. *Biochem. J.* **397,** 509–518.

Forbes, J. R., and Gros, P. (2001). Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol.* **9,** 397–403.

Fukui, Y., De Lozanne, A., and Spudich, J. A. (1990). Structure and function of the cytoskeleton of a Dictyostelium myosin-defective mutant. *J. Cell Biol.* **110,** 367–378.

Galdeen, S. A., Stephens, S., Thomas, D. D., and Titus, M. A. (2007). Talin influences the dynamics of the myosin VII-membrane interaction. *Mol. Biol. Cell* **18,** 4074–4084.

Gambino, M., Kay, R. R., and Bozzaro, S. (1992). Morphogenesis and differentiation of Dictyostelium cells interacting with immobilized glucosides - dependence on DIF production. *Differentiation* **49,** 133–141.

Garin, J., Diez, R., Kieffer, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001). The phagosome proteome: Insight into phagosome functions. *J. Cell Biol.* **152,** 165–180.

Gebbie, L., Benghezal, M., Cornillon, S., Froquet, R., Cherix, N., Malbouyres, M., Lefkir, Y., Grangeasse, C., Fache, S., Dalou, J., Bruckert, F., Letourneur, F. *et al.* (2004). Phg2, a kinase involved in adhesion and focal site modeling in Dictyostelium. *Mol. Biol. Cell* **15,** 3915–3925.

Gerisch, G. (1959). Ein Submerskulturverfahren fur entwicklungsphysiologische Untersuchungen an Dictyostelium discoideum. *Naturwissenschaften* **46,** 654–656.

Gotthardt, D., Warnatz, H. J., Henschel, O., Bruckert, F., Schleicher, M., and Soldati, T. (2002). High-resolution dissection of phagosome maturation reveals distinct membrane trafficking phases. *Mol. Biol. Cell* **13,** 3508–3520.

Gotthardt, D., Blancheteau, V., Bosserhoff, A., Ruppert, T., Delorenzi, M., and Soldati, T. (2006). Proteomic fingerprinting of phagosome maturation and evidence for the role of a Galpha during uptake. *Mol. Cell. Proteomics* **5,** 2228–2243.

Greenberg, S. (1995). Signal transducton of phagocytosis. *Trends Cell Biol* **5,** 93–99.

Gruenberg, J., and van der Goot, F. G. (2006). Mechanisms of pathogen entry through the endosomal compartments. *Nat. Rev. Mol. Cell Biol.* **7,** 495–504.

Gunshin, H. F. Y., Custodio, A. O., Direnzo, C., Robine, S., and Andrews, N. C. (2005). Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver. *J. Clin. Invest.* **115,** 1258–1266.

Haas, A. (2007). The phagosome: Compartment with a license to kill. *Traffic* **8,** 311–330.

Hacker, U., Albrecht, R., and Maniak, M. (1997). Fluid-phase uptake by macropinocytosis in Dictyostelium. *J. Cell Sci.* **110,** 105–112.

Haegele, S., Kohler, R., Merkert, H., Schleicher, M., Hacker, J., and Steinert, M. (2000). Dictyostelium discoideum: A new host model system for intracellular pathogens of the genus Legionella. *Cell. Microbiol.* **2,** 165–171.

Hagedorn, M., and Soldati, T. (2007). Flotillin and RacH modulate the intracellular immunity of Dictyostelium to Mycobacterium marinum infection. *Cell Microbiol.* **9,** 2716–2733.

Han, J. W., Leeper, L., Rivero, F., and Chung, C. Y. (2006). Role of RacC for the regulation of WASP and phosphatidylinositol 3-kinase during chemotaxis of Dictyostelium. *J. Biol. Chem.* **281,** 35224–35234.

Han, Y. H., Chung, C. Y., Wessels, D., Stephens, S., Titus, M. A., Soll, D. R., and Firtel, R. (2002). Requirement of a vasodilator-stimulated phosphoprotein family member for cell adhesion, the formation of filopodia, and chemotaxis in Dictyostelium. *J. Biol. Chem.* **277,** 49877–49887.

Harris, E., and Cardelli, J. (2002). RabD, a Dictyostelium Rab14-related GTPase, regulates phagocytosis and homotypic phagosome and lysosome fusion. *J. Cell Sci.* **115,** 3703–3713.

Harris, E., Wang, N., Wu, W., Weatherford, A., De Lozanne, A., and Cardelli, J. (2002). Dictyostelium LvsB mutants model the lysosomal defects associated with Chediak-Higashi syndrome. *Mol. Biol. Cell* **13,** 656–669.

Harrison, R. E., Brumel, J. H., Khandani, A., Bucci, C., Scott, C. C., Jiang, X., Finlay, B. B., and Grinstein, S. (2004). Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: Role of Rab7 and RILP. *Mol. Biol. Cell* **15,** 3146–3154.

Hellio, R., and Ryter, A. (1980). Relationships between anionic sites and lectin receptors in the plasma membrane of Dictyostelium discoideum and their role in phagocytosis. *J. Cell Sci.* **41,** 89–104.

Hemler, M. E. (2005). Tetraspanin functions and associated microdomains. *Nat. Rev. Mol. Cell Biol.* **6,** 801–811.

Hilbi, H., Segal, G., and Shuman, H. A. (2001). Icm/dot-dependent upregulation of phagocytosis by Legionella pneumophila. *Mol. Microbiol.* **42,** 603–617.

Hoeller, O., and Kay, R. R. (2007). Chemotaxis in the absence of PIP3 gradients. *Curr. Biol.* **17,** 813–817.

Ibarra, N., Pollitt, A., and Insall, R. H. (2005). Regulation of actin assembly by SCAR/WAVE proteins. *Biochem. Soc. Trans.* **33,** 1243–1246.

Iijima, M., and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* **109,** 599–610.

Insall, R., Muller-Taubenberger, A., Machesky, L., Kohler, J., Simmeth, E., Atkinson, S. J. Weber, I., and Gerisch, G. (2001). Dynamics of the Dictyostelium Arp2/3 complex in endocytosis, cytokinesis, and chemotaxis. *Cell Motil. Cytoskeleton.* **50,** 115–128.

Ishikawa, J., Okano, J., Ohki, K., Amagai, A., Maeda, Y., and Miyata, H. (2003). Phagocytosis of Dictyostelium discoideum studied by the particle-tracking method. *Exp. Cell Res.* **288,** 268–276.

Jabado, N., Patricia, C. -M., Grinstein, S., and Gros, P. (2003). Iron chelators modulate the fusogenic properties of Salmonella-containing phagosomes. *Proc. Natl. Acad. Sci. USA* **100,** 6127–6132.

Jenne, N., Rauchenberger, R., Hacker, U., Kast, T., and Maniak, M. (1998). Targeted gene disruption reveals a role for vacuolin B in the late endocytic pathway and exocytosis. *J. Cell Sci.* **111,** 61–70.

Jules, M., and Buchrieser, C. (2007). Legionella pneumophila adaptation to intracellular life and the host response: Clues from genomics and transcriptomics. *FEBS Lett.* **581,** 2829–2838.

Jung, G., Wu, X. F., and Hammer, J. A. (1996). Dictyostelium mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions. *J. Cell Biol.* **133,** 305–323.

Kagan, J. C., and Roy, C. (2002). Legionella phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat. Cell Biol.* **4,** 945–954.

Kessin, R. H. (2001). ''Dictyostelium - Evolution, cell biology, and the development of multicellularity.'' Cambridge Univ. Press, Cambridge, UK.

Khurana, T., Brzostowski, J. A., and Kimmel, A. R. (2005). A Rab21/LIM-only/CH-LIM complex regulates phagocytosis via both activating and inhibitory mechanisms. *EMBO J.* **24,** 2254–2264.

Kitano, M., Nakaya, M., Nakamura, T., Nagata, S., and Matsuda, M. (2008). Imaging of Rab5 activity identifies essential regulators for phagosome maturation. *Nature* **453,** 241–245.

Konzok, A., Weber, I., Simmeth, E., Hacker, U., Maniak, M., and Muller-Taubenberger, A. (1999). DAip1, a Dictyostelium homologue of the yeast actin-interacting protein 1, is involved in endocytosis, cytokinesis, and motility. *J. Cell Biol.* **146,** 453–464.

Kyei, G. B., Vergne, I., Chua, J., Roberts, E., Harris, J., Junutula, J. R., and Deretic, V. (2006). Rab14 is critical for maintenance of Mycobacterium tuberculosis phagosome maturation arrest.. *EMBO J.* **25,** 5250–5259.

Kypri, E., Schmauch, C., Maniak, M., and De Lozanne, A. (2007). The BEACH protein LvsB is localized on lysosomes and postlysosomes and limits their fusion with early endosomes. *Traffic* **8,** 774–783.

Lardy, B., Bof, M., Aubry, L., Paclet, M. H., Morel, F., Satre, M., and Klein, G. (2005). NADPH oxidase homologs are required for normal cell differentiation and morphogenesis in Dictyostelium discoideum. *Biochim. Biophys. Acta* **1744,** 199–212.

Larochelle, D. A., Vithalani, K. K., and De Lozanne, A. (1997). Role of Dictyostelium racE in cytokinesis: Mutational analysis and localization studies by use of green fluorescent protein. *Mol. Biol. Cell* **8,** 935–944.

Laurent, O., Bruckert, F., Adessi, C., and Satre, M. (1998). *In vitro* reconstituted Dictyostelium discoideum early endosome fusion is regulated by Rab7 but proceeds in the absence of ATP-Mg2+from the bulk solution. *J. Biol. Chem.* **273,** 793–799.

Lee, E., Pang, K., and Knecht, D. (2001). The regulation of actin polymerization and cross-linking in Dictyostelium. *Biochim. Biophys. Acta* **1525,** 217–227.

Lee, E., Seastone, D. J., Harris, E., Cardelli, J. A., and Knecht, D. A. (2003). RacB regulates cytoskeletal function in Dictyostelium spp. *Euk. Cell* **2,** 474–485.

Leippe, M., Bruhn, H., Hecht, O., and Groetzinger, J. (2005). Ancuent weapons: the three-dimensional structure of amoebapores A. *Trends Parasot* **21,** 5–7.

Leiva, N., Pavarotti, M., Colombo, M. I., and Damiani, M. T. (2006). Reconstitution of recycling from the phagosomal compartment in streptolysin O-permeabilized macrophages: Role of Rab11. *Exp. Cell Res.* **312,** 1843–1855.

Lemmon, M. (2003). Phosphoinositides recognition domains. *Traffic* **4,** 201–213.

Lemmon, M. (2007). Pleckstrin homology (PH9 domains and phosphoinositides. *Biochem. Soc. Symp.* **74,** 81–93.

Li, Z. R., Solomon, J. M., and Isberg, R. R. (2005). Dictyostelium discoideum strains lacking the RtoA protein are defective for maturation of the Legionella pneumophila replication vacuole. *Cell. Microbiol.* **7,** 431–442.

Little, K. D., Hemler, M. E., and Stipp, C. S. (2004). Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: Central role of CD81 in facilitating GPR56-Galpha q/11 association. *Mol. Biol. Cell* **15,** 2375–2387.

Loovers, H. M., Kortholt, A., de Groote, H., Whitty, L., Nussbaum, R. L., and van Haastert, P. J. (2007). Regulation pf phagocytosis in Dictyostelium by the inositol 5-phosphatase OCRL homolog Dd5P4. *Traffic* **8,** 618–628.

Lu, H., and Clarke, M. (2005). Dynamic properties of Legionella-containing phagosomes in Dictyostelium amoebae. *Cell. Microbiol.* **7,** 995–1007.

Malik, S., Abel, L., Tooker, H., Poon, A., Simkin, L., Girard, M., Adams, G. J., Starke, J. R., Smith, K. C., Graviss, E. A., Musser, J. M., and Schurr, E. (2005). Alleles of the NRAMP1 gene are risk factors for pediatric tuberculosis disease. *Proc. Natl. Acad. Sci. USA* **102,** 12183–12188.

Manahan, C. L., Iglesias, P. A., Long, Y., and Devreotes, P. N. (2004). Chemoattractant signaling in Dictyostelium discoideum. *Annu. Rev. Cell Dev. Biol.* **20,** 223–253.

Mangahas, P. M., Yu, X., Miller, K. G., and Zhou, Z. (2008). The small GTPase Rab2 functions in the removal of apoptotic cells in Caenorhabditis elegans. *J. Cell Biol.* **180,** 357–373.

Maniak, M. (2001). Fluid-phase uptake and transit in axenic Dictyostelium cells. *Biochim. Biophys. Acta* **1525,** 197–204.

Maniak, M. (2002). Conserved features of endocytosis in Dictyostelium. *Int. Rev. Cytol.* **221,** 257–287.

Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J., and Gerisch, G. (1995). Coronin involved in phagocytosis: Dynamics of particle-induced relocalization visualized by a green fluorescent protein tag. *Cell* **83,** 915–924.

Maselli, A., Laevsky, G., and Knecht, D. A. (2002). Kinetics of binding, uptake and degradation of live fluorescent (DsRed) bacteria by Dictyostelium discoideum. *Microbiology* **148,** 413–420.

Mesa, R., Salomón, C., Roggero, M., Stahl, P. D., and Mayorga, L. S. (2001). Rab22a affects the morphology and function of the endocytic pathway. *J. Cell Sci.* **114,** 4041–4049.

Minakami, R., and Sumimotoa, H. (2006). Phagocytosis-coupled activation of the superoxide-producing phagocyte exidase, a member of the NADPH oxidase (nox) family. *Int. J. Hematol.* **84,** 193–198.

Mueller, P., and Pieters, J. (2006). Modulation of macrophage antimicrobial mechanisms by pathogenic mycobacteria. *Immunobiology* **211,** 549–556.

Mueller, P., Subert, N., Otto, H., Herbst, R., Ruehling, H., Maniak, M., and Leippe, M. (2005). A Dictyostelium mutant with reduced lysozyme levels compensates by increased phagocytic activity. *J. Biol. Chem.* **280,** 10435–10443.

Muller-Taubenberger, A., Lupas, A. N., Li, H. W., Ecke, M., Simmeth, E., and Gerisch, G. (2001). Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *EMBO J.* **20,** 6772–6782.

Myers, S. A., Han, J. W., Lee, Y., Firtel, R. A., and Chung, C. Y. (2005). A Dictyostelium homologue of WASP is required for polarized F-actin assembly during chemotaxis. *Mol. Biol. Cell* **16,** 2191–2206.

Nagai, H., Kagan, J., Zhu, X., Kahn, R. A., and Roy, C. R. (2002). A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes. *Science* **295,** 679–682.

Neuhaus, E. M., Almers, W., and Soldati, T. (2002). Morphology and dynamics of the endocytic pathway in Dictyostelium discoideum. *Mol. Biol. Cell* **13,** 1390–1407.

Nevo, Y., and Nelson, N. (2006). The Nramp family pf metal-ion transporters. *Biochim. Biophys. Acta* **1763,** 609–620.

Niewohner, J., Weber, I., Maniak, M., Muller-Taubenberger, A., and Gerisch, G. (1997). Talin-null cells of Dictyostelium are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. *J. Cell Biol.* **138,** 349–361.

Noegel, A. A., and Schleicher, M. (2000). The actin cytoskleleton of Dictyostelium: A story told by mutants. *J. Cell Sci.* **113,** 759–766.

Nolta, K. V., Padh, H., and Steck, T. L. (1991). Acidosomes from Dictyostelium - initial biochemical characterization. *J. Biol. Chem.* **266,** 18318–18323.

Padh, H., Lavasa, M., and Steck, T. L. (1989). Prelysosomal acidic vacuoles in Dictyostelium discoideum. *J. Cell Biol.* **108,** 865–874.

Padh, H., Ha, J. H., Lavasa, M., and Steck, T. L. (1993). A post-lysosomal compartment in Dictyostelium discoideum. *J. Biol. Chem.* **268,** 6742–6747.

Payrastre, B., Missy, K., Giurato, S., Bodin, S., Plantavid, M., and Gratacap, M. (2001). Phopshoinositides: Key players in cell signalling, in time and space. *Cell Signal.* **13,** 377–387.

Peracino, B., Borleis, J., Jin, T., Westphal, M., Schwartz, J. M., Wu, L. J., Bracco, E., Gerisch, G., Devreotes, P., and Bozzaro, S. (1998). G protein beta subunit-null mutants are impaired in phagocytosis and chemotaxis due to inappropriate regulation of the actin cytoskeleton. *J. Cell Biol.* **141,** 1529–1537.

Peracino, B., Wagner, C., Balest, A., Balbo, A., Pergolizzi, B., Noegel, A. A., Steinert, M. and Bozzaro, S. (2006). Function and mechanism of action of Dictyostelium Nramp1 (Slc11a1) in bacterial infection. *Traffic* **7,** 22–38.

Prabhu, Y., and Eichinger, L. (2006). The Dictyostelium repertoire of seven transmembrane domain receptors. *Eur. J. Cell Biol.* **85,** 937–946.

Pradel, E., and Ewbank, J. J. (2004). Genetic models in pathogenesis. *Annu Rev Genet.* **38,** 347–363.

Pukatzki, S., Kessin, R. H., and Mekalanos, J. J. (2002). The human pathogen Pseudomonas aeruginosa utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. *Proc. Natl. Acad. Sci. USA* **99,** 3159–3164.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. S. F., and Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. *Proc. Natl. Acad. Sci. USA* **103,** 1528–1533.

Rauchenberger, R., Hacker, U., Murphy, J., Niewohner, J., and Maniak, M. (1997). Coronin and vacuolin identify consecutive stages of a late, actin-coated endocytic compartment in Dictyostelium. *Curr. Biol.* **7,** 215–218.

Ravanel, K., de Chassey, B., Cornillon, S., Benghezal, M., Zulianello, L., Gebbie, L., Letourneur, F., and Cosson, P. (2001). Membrane sorting in the endocytic and phagocytic pathway of Dictyostelium discoideum. *Eur. Cell Biol.* **80,** 754–764.

Rezabek, B. L., Rodriguez-Paris, J. M., Cardelli, J. A., and Chia, C. P. (1997). Phagosomal proteins of Dictyostelium discoideum. *J. Euk. Microbiol.* **44,** 284–292.

Richer, E., Courville, P., Bergevin, I., and Cellier, M. F. (2003). Horizontal gene transfer of ''prototype'' Nramp in bacteria. *J. Mol. Evol.* **57,** 363–376.

Ridley, A. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol.* **16,** 522–529.

Rivero, F., Koppel, B., Peracino, B., Bozzaro, S., Siegert, F., Weijer, C. J., Schleicher, M., Albrecht, R., and Noegel, A. A. (1996). The role of the cortical cytoskeleton: F-actin crosslinking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. *J. Cell Sci.* **109,** 2679–2691.

Rivero, F., Albrecht, R., Dislich, H., Bracco, E., Graciotti, L., and Bozzaro, S. (1999). RacF1, a novel member of the Rho protein family in Dictyostelium discoideum, associates transiently with cell contact areas, macropinosomes, and phagosomes. *Mol. Biol. Cell* **10,** 1205–1219.

Roberts, R. L., Barbieri, M. A., Ullrich, J., and Stahl, P. D. (2000). Dynamics of rab5 activation in endocytosis and phagocytosis. *J. Leukoc. Biol.* **174,** 923–929.

Roberts, E. A., Chua, J., Kyei, G. B., and Deretic, V. (2006). Higher order Rab programming in phagolysosome biogenesis. *J. Cell Biol.* **174,** 923–929.

Rodriguez-Paris, J. M., Nolta, K. V., and Steck, T. L. (1993). Characterization of lysosomes isolated from Dictyostelium discoideum by magnetic fractionation. *J. Biol. Chem.* **268,** 9110–9116.

Rupper, A., and Cardelli, J. (2001). Regulation of phagocytosis and endo-phagosomal trafficking pathways in Dictyostelium discoideum. *Biochim. Biophys. Acta* **1525,** 205–216.

Rupper, A., Grove, B., and Cardelli, J. (2001a). Rab7 regulates phagosome maturation in Dictyostelium. *J. Cell Sci.* **114,** 2449–2460.

Rupper, A. C., Rodriguez-Paris, J. M., Grove, B. D., and Cardelli, J. A. (2001b). p110-related PI 3-kinases regulate phagosome-phagosome fusion and phagosomal pH through a PKB/Akt dependent pathway in Dictyostelium. *J. Cell Sci.* **114,** 1283–1295.

Sasaki, A. T., and Firtel, R. A. (2006). Regulation of chemotaxis by the orchestrated activation of Ras, PI3K, and TOR. *Eur. J. Cell Biol.* **85,** 873–895.

Schreiner, T., Mohrs, M. R., Blau-Wasser, R., von Krempelhuber, A., Schleicher, M., and Noegel, A. A. (2002).*Euk Cell* **1,** 906–914.

Schreiner, T., Mohrs, M. R., Blau-Wasser, R., von Krempelhuber, A., Steinert, M., Schleicher, M., and Noegel, A. A. (2003). Loss of the F-actin binding and vesicle-associated protein comitin leads to a phagocytosis defect. *Euk. Cell* **1,** 906–914.

Schwarz, E. C., Neuhaus, E. M., Kistler, C., Henkel, A. W., and Soldati, T. (2000). Dictyostelium myosin IK is involved in the maintenance of cortical tension and affects motility and phagocytosis. *J. Cell Sci.* **113,** 621–633.

Scott, C. C., Dobson, W., Botelho, R. J., Coady-Osberg, N., Chavrier, P., Knecht, D. A., Heath, C., Stahl, P., and Grinstein, S. (2005). Phosphatidylinositol-4,5-bisphosphate hydrolysis directs actin remodeling during phagocytosis. *J. Cell Biol.* **169,** 139–149.

Seastone, D. J., Lee, E., Bush, J., Knecht, D., and Cardelli, J. (1998). Overexpression of a novel Rho family GTPase, RacC, induces unusual actin-based structures and positively affects phagocytosis in Dictyostelium discoideum. *Mol. Biol. Cell* **9,** 2891–2904.

Seastone, D. J., Zhang, L. Y., Buczynski, G., Rebstein, P., Weeks, G., Spiegelman, G., and Cardelli, J. (1999). The small Mr Ras-like GTPase Rap1 and the phospholipase C pathway act to regulate phagocytosis in Dictyostelium discoideum. *Mol. Biol. Cell* **10,** 393–406.

Seastone, D. J., Harris, E., Temesvari, L. A., Bear, J. E., Saxe, C. L., and Cardelli, J. (2001). The WASp-like protein Scar regulates macropinocytosis, phagocytosis and endosomal membrane flow in Dictyostelium. *J. Cell Sci.* **114,** 2673–2683.

Sillo, A., Bloomfield, G., Balest, A., Balbo, A., Pergolizzi, B., Peracino, B., Skelton, J., Ivens, A., and Bozzaro, S. (2008). Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in Dictyostelium. *BMC Genomics* **9,** 291.

Simpson, J. C., Griffiths, G., Wessling-Resnick, M., Fransen, J. A., Bennett, H., and Jones, A. T. (2004). A role for the small GTPase Rab21 in the early endocytic pathway. *J. Cell Sci.* **117,** 6297–6311.

Skriwan, C., Fajardo, M., Hagele, S., Horn, M., Wagner, M., Michel, R., Krone, G., Schleicher, M., Hacker, J., and Steinert, M. (2002). Various bacterial pathogens and symbionts infect the amoeba Dictyostelium discoideum. *Int. J. Med. Microbiol.* **291,** 615–624.

Smith, A. C., Heo, W. D., Braun, V., Jiang, X., Macrae, C., Casanova, J. E., Scidmore, M. A., Grinstein, S., Meyer, T., and Brumell, J. H. (2007). A network of Rab GTPases controls phagosome maturation and is modulated by Salmonella enterica serovar Typhimurium. *J. Cell Biol.* **176,** 263–268.

Soldati, T. (2003). Unconventional myosins, actin dynamics and endocytosis: A menage a trois? *Traffic* **4,** 358–366.

Solomon, J. M., and Isberg, R. R. (2000). Growth of Legionella pneumophila in Dictyostelium discoideum: A novel system for genetic analysis of host-pathogen interactions. *Trends Microbiol.* **8,** 478–480.

Solomon, J. M., Rupper, A., Cardelli, J. A., and Isberg, R. R. (2000). Intracellular growth of Legionella pneumophila in Dictyostelium discoideum, a system for genetic analysis of host-pathogen interactions. *Inf. Immun.* **68,** 2939–2947.

Solomon, J. M., Leung, G. S., and Isberg, R. R. (2003). Intracellular replication of Mycobacterium marinum within Dictyostelium discoideum: Efficient replication in the absence of host coronin. *Inf. Immun.* **71,** 3578–3586.

Somesh, B. P., Neffgen, C., Iijima, M., Devreotes, P., and Rivero, F. (2006a). Dictyostelium RacH regulates endocytic vesicular trafficking and is required for localization of vacuolin. *Traffic* **7,** 1194–1212.

Somesh, B. P., Vlahou, G., Iijima, M., Insall, R. H., Devreotes, P., and Rivero, F. (2006b). RacG regulates morphology, phagocytosis and chemotaxis. *Euk. Cell* **5,** 1648–1663.

Souza, G. M., Mehta, D. P., Lammertz, M., Rodriguez-Paris, J., Wu, R. R., Cardelli, J. A., and Freeze, H. H. (1997). Dictyostelium lysosomal proteins with different sugar modifications sort to functionally distinct compartments. *J. Cell Sci.* **110,** 2239–2248.

Stauffer, T. P., Ahn, S., and Meyer, T. (1998). Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration moitored in living cells. *Curr. Biol.* **8,** 343–346.

Steenbergen, J. N., Nosanchuk, J. D., Malliaris, S. D., and Casadevall, A. (2003). Cryptococcus neoformans virulence is enhanced after growth in the genetically malleable host Dictyostelium discoideum. *Inf. Immun.* **71,** 4862–4872.

Steimle, P. A., Yumura, S., Cote, G. P., Medley, Q. G., Polyakov, M. V., and Leppert, B. (2001). Recruitment of a myosin heavy chain kinase to actin-rich protrusions in Dictyostelium. *Curr. Biol.* **11,** 708–713.

Steinert, M., and Heuner, K. (2005). Dictyostelium as host model for pathogenesis. *Cell. Microbiol.* **7,** 307–314.

Stuart, L. M., and Ezekowitz, R. A. B. (2005). Phagocytosis elegant complexity. *Immunity* **22,** 539–550.

Stuart, L. M., and Ezekowitz, R. A. B. (2008). Phagocytosis and comparative innate immunity: Learning on the fly. *Nature Rev. Immunol* **8,** 131–141.

Sturgill-Koszycki, S., and Swanson, M.S (2000). Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles. *J. Exp. Med.* **192,** 1261–1272.

Sturgill-Koszycki, S., Schlesinger, P., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J., and Russell, D. G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263,** 637–639.

Su, M. A., Trenor, C., Fleming, J. C., Fleming, M. D., and Andrews, N. C. (1998). The G185R mutation disrupts function of the iron transporter Nramp2. *Blood* **92,** 2157–2163.

Sultana, H., Rivero, F., Blau-Wasser, R., Schwager, S., Balbo, A., Bozzaro, S., Schleicher, M., and Noegel, A. A. (2005). Cyclase-associated protein is essential for the functioning of the endo-lysosomal system and provides a link to the actin cytoskeleton. *Traffic* **6,** 930–946.

Swanson, J., and Baer, S. C. (1995). Phagocytosis by zippers and triggers. *Trends Cell Biol.* **5,** 89–93.

Swanson, M., and Isberg, R. R. (1995). Association of Legionella pneumophyla with the macrophage endoplasmic reticulum. *Infect. Immun.* **63,** 3609–3620.

Temesvari, L., Rodriguez-Paris, J., Bush, J., Steck, T. L., and Cardelli, J. (1994). Characterization of lysosomal membrane proteins of Dictyostelium discoideum - A complex population of acidic integral membrane glycoproteins, Rab GTP-binding proteins, and vacuolar ATPase subunits. *J. Biol. Chem.* **269,** 25719–25727.

Temesvari, L., Zhang, L. Y., Fodera, B., Janssen, K. P., Schleicher, M., and Cardelli, J. A. (2000). Inactivation of lmpA, encoding a LIMPII-related endosomal protein, suppresses the internalization and endosomal trafficking defects in profilin-null mutants. *Mol. Biol. Cell* **11,** 2019–2031.

Titus, M. A. (1999). A class VII unconventional myosin is required for phagocytosis. *Curr. Biol.* **9,** 1297–1303.

Tuxworth, R. I., Weber, I., Wessels, D., Addicks, G. C., Soll, D. R., Gerisch, G., and Titus, M. A. (2001). A role for myosin VII in dynamic cell adhesion. *Curr. Biol.* **11,** 318–329.

Vidal, S. M., Malo, D., Vogan, K., Skamene, E., and Gros, P. (1993). Natural resistance to infection with intracellular parasites: Isolation of a candidate for Bcg. *Cell* **73,** 469–485.

Vieira, O. V., Bucci, C., Harrison, R. E., Trimble, W. S., Lanzetti, L., Gruenberg, J., Schreiber, A. D., Stahl, P. D., and Grinstein, S. (2003). Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Mol. Cell Biol.* **23,** 2501–2514.

Vlahou, G., and Rivero, F. (2006). Rho GTPase signaling in Dictyostelium discoideum: Insights from the genome. *Eur. J. Cell Biol.* **85,** 947–959.

Vogel, G., Thilo, L., Schwarz, H., and Steinhart, R. (1980). Mechanism of phagocytosis in Dictyostelium discoideum: Phagocytosis is mediated by different recognition sites as disclosed by mutants with altered phagocytotic properties. *J. Cell Biol.* **86,** 456–465.

Vogel, J. P., Andrews, H. L., Wong, S. K., and Isberg, R. R. (1998). Conjugative transfer by the virulence system of Legionella pneumophila. *Science* **279,** 873–876.

Waddell, D. R., and Duffy, K. T. I. (1986). Breakdown of self/nonself recognition in cannibalistic strains of the predatory slime mold, Dictyostelium caveatum. *J. Cell Biol.* **102,** 298–305.

Weber, S. S., Ragaz, C., Reus, K., Nyfeler, Y., and Hilbi, H. (2006). Legionella pneumophila exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS Pathog.* **2,** e46.

Weeks, G., and Spiegelman, G. B. (2003). Roles played by Ras subfamily proteins in the cell and developmental biology of microorganisms. *Cell. Signal.* **15,** 901–909.

Willard, S. S., and Devreotes, P. N. (2006). Signaling pathways mediating chemotaxis in the social amoeba Dictyostelium discoideum. *Eur. J. Cell Biol.* **85,** 897–904.

Wu, L. J., and Devreotes, P. N. (1991). Dictyostelium transiently expresses eight distinct G-protein alpha-subunits during its developmental program. *Biochem. Biophys. Res. Commun.* **179,** 1141–1147.

Yeung, T., and Grinstein, S. (2007). Lipid signaling and the modulation of surface charge during phagocytosis. *Immunol. Rev.* **219**.

Yumura, S., and Uyeda, T. Q. (2003). Myosins and cell dynamics in cellular slime molds. *Intern. Rev. Cytol.* **224,** 173–225.

Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. *Nature Rev. Cell. Mol. Biol.* **2,** 107–117.

Zhou, K. M., Takegawa, K., Emr, S. D., and Firtel, R. A. (1995). A phosphatidylinositol (PI) kinase gene family in Dictyostelium discoideum: Biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development. *Mol. Cell. Biol.* **15,** 5645–5656.

Zhou, K., Pandol, S., Bokoch, G., and Traynor-Kaplan, A. E. (1998). Disruption of Dictyostelium PI3K genes reduces [32P]phosphatidylinositol 3,4 bisphosphate and [32P]phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis. *J. Cell Sci.* **111,** 283–294.

Zouwail, S., Pettitt, T. R., Dove, S., Chibalina, M., Powner, D. J., Haynes, L., Wakelan, M. J., and Insall, R. H. (2005). Phospholipase D activity is essential for actin localization and actin-based motility in Dictyostelium. *Biochem. J.* **389,** 207–214.